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(54) Title: METHOD FOR GENERATING HIGHLY ACTIVE HUMAN DENDRITIC CELLS FROM MONOCYTES

(57) Abstract: The present invention relates to a process for deriving dendritic cells from mononuclear cells in culture comprising the step of putting in contact type I IFN with said mononuclear cells. Dendritic cells suitable as cellular adjuvants in prophylactic as well as therapeutic vaccination of animal and human beings, are obtainable thereby, after a single step treatment in a brief period of time. Dendritic cells obtainable thereby, pharmaceutical compositions including them, in particular a vaccine comprising said cells as active principle, and a method of treatment of a pathology associated with the presence of an antigen in human beings, are further objects of the invention, as well as a kit for deriving said dendritic cells and a method for the ex vivo expansion of T cells using them.

METHOD FOR GENERATING HIGHLY ACTIVE HUMAN DENDRITIC CELLS FROM MONOCYTES.

DESCRIPTION

Field of the invention

The present invention relates to the field of immunotherapy, and in particular to vaccines including as an adjuvant human or animal dendritic cells.

Background of the invention

Dendritic cells (DCs) are known in the art. In absence of lineage specific markers, they are generally identified by the lack of leukocyte markers of other lineages (CD3 for T cell lineage, CD14 and CD15 for monocytic and granulocyte lineages, CD19, CD20 and CD24 for B cell lineage and CD16, CD56 and CD57 for NK cell lineage) by their specific immunophenotype (positive for surface antigen CD40, CD80, CD86), and their morphology (characterized by the presence of dendrites or membrane processes) (1-3).

From the functional point of view, DCs are known to be highly potent antigen-presenting cells (APC), playing *in vivo* a pivotal role in the priming of the immune response (1-3). In this connection, a main distinction is made between mature and immature DCs.

Immature DCs are weak initiators of immune response specialized in capturing and processing antigens, phenotypically characterized by low expression of the accessory molecules CD40, CD80, CD86 and the lack of CD83 expression. Upon appropriate stimuli, DCs undergo extensive changes: loss of antigen-capturing function and the upregulation of the expression of costimulatory molecules (CD40, CD80 and CD86) together with the induction of CD83 and CD25(1-4).

Terminally differentiated/mature DCs are instead capable of readily priming naive T cells within lymphoid tissues.

Phenotype of DCs in the mature state is characterized accordingly by the production of a variety of cytokines, including typically IL-15, (1-3, 5, 6) which are considered capable to affect, by autocrine/paracrine mechanisms, the phenotype and functional activity of DCs themselves as well as of other host cells (7-9).

Phenotype of mature/activated DCs is also characterized by specific chemotactic properties. In this connection, it is well known in the art that migration of DCs is tightly regulated as a function of maturation (10-13).

Thus, immature DCs respond to inflammatory chemokines, such as MIP-1α, MIP-1β, RANTES and MIP-3α (14) as a consequence of the expression of the chemokine receptors CCR5 and CCR6, while mature DCs have lost their responsiveness to most of these chemokines, as a result of down-regulation of cognate receptor expression or activity (15).

Conversely, mature DCs have been reported to respond to MIP-3β/ELC and 6Ckine/SLC as a consequence of the induction of their specific receptor CCR7 which is lacking on immature DCs (10, 11, 15).

On the other hand, DCs are themselves producer of a series of chemokines. Upon maturation, DCs have an initial burst of Mip-3α, Mip-3β and IL-8, whereas RANTES and MCP-1 are produced in a more sustained fashion. The production of MIP-3β/ELC by activated/mature DCs is also important in supporting the generation of the immune response by

recruiting naive T and B cells, which selectively express CCR7.

Mature DCs express also IP-10, a potent chemoattractant for activated/memory Th1 cells by binding to the receptor CXCR3 (10, 16, 17), while immature DCs express MDC and TARC attracting specifically chronically activated Th2 lymphocytes. (10, 18). In addition, in presence of mature DCs and IL-12, T-helper cells turn into IFNγ-producing Th1 cells, which promote the cellular arm of the immune response, whereas $CD8^+$ cytotoxic T cells are induced to proliferate vigorously. IFNγ and IL-12 promote further the differentiation of T cells into killer cells.

Accordingly, mature DCs are considered capable of stimulating the outgrowth and activation of a variety of T cells.

The ability to prime antigen-specific naive T cells represent a unique and critical function of DCs. Moreover, by virtue of their enhanced expression of HLA and costimulatory molecules, DCs stimulate allogeneic MLR (which allows comparison of the capacity of different APCs to stimulate T cell proliferation independently of the antigen) more efficiently than any other antigen presenting cell. Thus, there is a growing interest in utilizing such cells as cellular adjuvants for prophylactic or therapeutic vaccination toward infectious agents or tumors.

However, the use of DCs has been limited by their very low frequency in peripheral blood and the invasiveness of procedures aimed to gain access to bone marrow or lymphoid organs. Such limitations render complicate and expensive obtaining DCs to be used as adjuvant and application related thereto.

Consequently, some processes allowing production of DCs *in vitro* have been defined. These procedures are all based on the information that DCs originate from progenitor $CD34^+$ cells in bone marrow and blood or can be derived from peripheral blood mononuclear $CD14^+$ cells (19, 20). Hence, according to a first approach DCs are generated by cultivation of $CD34^+$ progenitors in medium containing Flt3-L or SCF (stem cell factor), followed by a combinations of various cytokines including GM-CSF, IL-4, and TNFα (3, 4).

In a second approach, an initial phase of cultivation of progenitors $CD34^+$ cells is carried out in the presence of GM-CSF, TNF-α and IL-4 (PCT/AU97/00801) followed by treatment with type I IFN.

Following a further approach, $CD34^+$ precursor cells from cord blood or bone marrow are cultivated in presence of IL-3 or GM-CSF (21). Thus, this procedure has been shown to induce cell proliferation, which is strongly potentiated by TNFα and culminates in the appearance of $CD1a^+$ cells displaying typical DC morphology and surface markers. $CD34^+$ precursor cells cultured in the presence of GM-CSF and TNFα differentiate into two distinct DC populations within 5-7 days, as defined by the exclusive expression of CD14 and CD1a. However, by further culturing, CD1a expression is generally downregulated just as CD83 appears (3).

According to a fourth approach, immature DCs are generated starting from peripheral blood $CD14^+$ monocytes cultivated in GM-CSF in conjunction with IL-13 or IL-4 for 5-7 days. DCs produced according to this procedure, however, display features of and behave as immature DCs expressing low levels of CD80 and CD86. Consequently, these DCs act as weak stimulators of a specific T cell response

and MLR. In this setting, further DC maturation can be driven by the addition of TNFα, IL-1, LPS, monocyte-conditioned medium (22) or sCD40L for two additional days (2, 3).

Thus, the requirement of a further step for DC maturation by addition of other factors to immature DCs represents a strong limitation for the rapid generation of DCs highly effective for clinical purposes. Moreover, it is not clear whether the use of mature DCs represents an advantage over immature DCs for clinical applications. In this context, DCs endowed with intermediate phenotypic and functional properties, i.e.: high phagocytic activity associated to the expression of membrane markers typical of mature DCs and to a potent immunostimulatory capacity, would represent a novel cellular entity of great interest for clinical applications.

Summary of the invention

Object of the present invention is to provide a process which allows a rapid generation of partially mature and highly functional DCs, suitable for use in prophylactic as well as therapeutic vaccination of animal and human beings.

Such an object is achieved according to a first aspect of the present invention by a process for deriving dendritic cells from mononuclear cells in culture, comprising the step of putting in contact said mononuclear cells with type I interferon at a final concentration greater than 100 IU /ml, since the initial culture thereof.

A first advantage of the process of the invention is given in that partially mature DCs are obtainable thereby from freshly isolated monocytes after a single step treatment including type I IFN as an essential factor.

A second advantage of the process of the invention is that it provides a particularly rapid procedure for DC production which can be carried out in a brief period of time (within three days of culture).

A third and main advantage is the generation of highly stable and partially mature DCs. Such DCs are endowed with more powerful "in vitro" and "in vivo" activities than those exhibited by DCs obtainable by the procedure known in the art.

In this connection the process of the invention is preferably carried out within three days of culture and more preferably, in presence of a growth factor, such as GM-CSF (Granulocyte/Monocyte-Colony Stimulating Factor) or the like, which promotes monocyte/DC survival in culture.

The GM-CSF is used preferably at a concentration in a range of 250-1,000 U/ml.

Type I IFN suitable in the process of the invention can be selected from the group consisting of any natural IFNα, any recombinant species or subtype of IFNα, consensus IFN (IFNcon1, herein named also CIFN), natural or recombinant IFNβ, and any synthetic type I IFN.

As reported above, IFN shall preferably be present in the culture medium at a final concentration greater than 100 IU /ml. Preferred embodiments in this connection are, however the ones wherein type I IFN is present in a concentration comprised in a range of 100-10,000 IU /ml, or more preferably in a range of 400-10,000 IU /ml, or 500-2,000 IU /ml, particularly 1,000 IU /ml. Using the latter range and concentration, DCs acquire the optimal expression of membrane markers associated with functional activity, with minimal toxic effects and good cell viability.

Since the GM-CSF is a constant culture component for monocyte-derived DCs, effects of differentiation are ascribed to IFN for DC populations originated in presence of IFN/GM-CSF (herein also defined IFN-DCs).

Mononuclear cells particularly preferred in the process of the invention are isolated from peripheral blood mononuclear cells (PBMC), and particularly $CD14^+$ monocytes, in an embodiment which has the further advantage of employing an easily available starting product. Alternatively, total unseparated or adherent PBMC are utilized in the procedure described.

The cells can be cultured in any medium suitable for culturing DCs "in vitro". In the specific case of treatment of human patients, culture media like X-VIVO 20 or AIM-V, are preferably used.

In a further preferred embodiment the process of the invention comprises also the step of putting in contact the cells that have been treated with type I IFN with a maturation agent. Such an embodiment is particularly suitable in all the cases, which can be identified by a skilled person, wherein a further maturation of the DCs obtained by treating the mononuclear cells with type I IFN as reported above is desired.

As reported above, a main advantage of the process of the invention is that it allows the production of DCs which are highly stable and functional.

Such DCs, as described in details below, exhibit in fact an immunophenotype, morphology, chemotaxis, and immunological activity not present in DCs derived according to other processes known in the art. Such features render DCs of the invention particularly suitable as an adjuvant in vaccine administration.

A further object of the present invention are DCs obtainable by the process of the invention, which in a preferred embodiment are loaded with antigenic peptides or proteins, or with a cellular extract containing at least one antigen, or with nucleic acids, as well as a pharmaceutical composition and vaccines including said DCs as an adjuvant or as an active principle , together with at least one antigen and a pharmaceutical acceptable carrier vehicle or auxiliary agent, and a process for producing said pharmaceutical compositions and vaccines.. The antigen(s), may be a viral, bacterial or tumor antigen, whose presence of which is associated with a pathology.

Any vehicle, carrier, auxiliary agent and formulation adopted in the art of vaccines production can be used in the vaccine of the invention. A skilled person can identify said components and all the steps of the relevant process of manufacturing.

A still further object of the present invention are a pharmaceutical compositions or vaccines comprising the DCs of the invention together with a pharmaceutically acceptable carrier, vehicle or auxiliary agent, said carrier vehicle and auxiliary agent being identifiable by a person skilled in the art.

The vaccines and compositions of the invention preferably comprise DCs loaded with specific antigens. Alternatively, the vaccines and compositions of the invention may comprise Dcs that have not been previously incubated with antigens. In the latter case, once the composition or vaccine is injected into a subject in need thereof, the antigens are locally acquired by the DCs.

The above described vaccines and pharmaceutical compositions are suitable according to the invention for use in a method for the prophylaxis or the therapy of a

pathology associated with the presence of an antigen in the human body..Said pathology is preferablyan infection or a neoplastic disease. Even more preferably said pathology is a lymphoma, a viral infection such as a HIV, HBV or HCV infection or a virally induced neoplastic diseases such as a neoplastic disease induced by Epstein Barr virus.The invention also relates to a method for the prophylaxis or treatment of a pathology associated with the presence of an antigen in the human body, comprising the administration to a subject in need thereof of an appropriate amount of monocyte-derived dendritic cells of the invention, preferably in an amount comprised between about 10^7 and about $5x10^9$ and preferably between about 10^8 and about 10^9.

The DCs of the invention can also be used for the ex vivo expansion of T cells, which can be CD4+ and/or CD8+ or both, in a method for the ex vivo expansion of T cells comprising the step of putting in contact said T cells with the dendritic cells of the invention. T cells so treated can be administered to humans for treating immune disorders or deterioration.

Object of the present invention are also kits containing means for the preparation of DCs.

A better description of the invention will be given with the help of the annexed figures.

Description of the figures

Figure 1, shows the dot histogram analysis of the immunophenotype of DCs obtained by treating blood-derived $CD14^+$ monocytes for three days with 1,000 IU /ml of IFNαn (natural IFNα, Alfa-Wasserman) and 500 U/ml of GM-CSF (IFN-DCs) as compared to DCs obtained by treating monocytes with 500 U/ml of IL-4 and 500 U/ml of GM-CSF (IL-4-DCs) for

three days. Monocytes were purified by standard Ficoll and 46% Percoll density gradient centrifugations followed by positive immunomagnetic sorting for $CD14^+$ cells (purity >95%). Monocytes were resuspended at the concentration of 2 x 10^6 cell/ml and treated as described in details in "Description of the invention" for 3 days. After staining with fluorochrome-conjugated monoclonal antibodies to cellular membrane markers, the cells were analyzed by flow cytometry, elettronically gating DCs according to light scatter properties, in order to exclude contaminating lymphocytes and cell debris. Data were acquired and analyzed using a FACSort flow cytometer and "Cell Quest" software (Becton Dickinson). The diagrams in the figure show the expression of a series of membrane markers in IFN-DCs (panel A) and IL-4-DCs (panel B). In each diagram, the *x* axis represents the cell fluorescence intensity relative to the analyzed marker, whereas the *y* axis represents the number of positive cells. Dotted lines represent the staining with isotype matched control antibodies to an irrelevant antigen.

Figure 2 shows comparative dot histogram profiles of DCs obtained from monocyte-enriched PBMCs treated with 1,000 IU /ml of different type I IFN preparations and 500 U/ml of GM-CSF, for three days. Monocyte fraction was enriched by standard Ficoll density gradient centrifugation and subsequent centrifugation on 46% Percoll density gradient of blood-derived PBMCs. After partial purification, the cell suspension contained <35% of contaminating lymphocytes. After staining with fluorochrome-conjugated monoclonal antibodies to surface markers, DCs were elettronically gated according to light scatter properties and analyzed by flow cytometry as described in figure 1. In each diagram, showing the expression of specific surface markers, the *x* axis

represents the cell fluorescence intensity, whereas the *y* axis represents the cell count. Control staining profiles were all within the first logaritmic decade of fluorescence intensity.

Figure 3 shows a diagram comparing the effects of different doses of type I IFN, in particular 1,000 IU /ml, 500 IU /ml and 100 IU /ml, added together with 500 U/ml of GM-CSF, on the expression of costimulatory molecules. Freshly isolated monocytes were partially purified by Ficoll and Percoll density centrifugations, cultured with cytokines and analyzed for antigen expression on day 3, by flow cytometry. Representative data from one out of three experiments are shown. Bars represents the mean fluorescence intensity values of selected DC membrane antigens as indicated in the figure.

Figure 4. Immunocytochemistry for CD44 expression in DCs generated in the presence of type I IFN (a) as compared to IL-4-DCs (b) (PAP/AEC and haematoxilyn counterstaining; magnification 1500x). The photos show the typical morphology of IFN/GM-CSF- and IL-4/GM-CSF-induced DCs. Note the thin and long dendrites of IFN-derived DCs as compared to the squat dendrites of the IL-4-DCs. Notably, the CD44 staining is typically localized on dendrites, nicely outlining them.

Figure 5. Panel A shows RT-PCR analysis of cytokine mRNA expression in DCs generated in the presence of either type I IFN and GM-CSF or IL-4/GM-CSF for 3 days. RT-PCR was performed as described in the examples. Panel A shows photographs of PCR products, derived from specific amplification of different mRNAs, as evidenced by transillumination of 1.5% agarose electrophoresis gels stained with ethidium bromide.

Panel B shows secretion of IL-15 in DC culture supernatant. Histograms represent the concentration of IL-15 protein, as assessed by ELISA, in supernatants from DCs generated in the presence of 1,000 IU /ml of different type I IFN preparations and in the presence of 500 U/ml of IL-4, in all cases in conjunction with 500 U/ml of GM-CSF for 3 days. Values are expressed as mean of 3 experiments ± S.D.

Figure 6, panel A shows a comparative MLR assay with DCs generated in the presence of various preparations of type I IFN and GM-CSF or IL-4/GM-CSF. Allogeneic PBLs were stimulated by DCs (at different stimulator/responder ratio) previously cultured for 3 days with IFN/GM-CSF or IL-4/GM-CSF. Lymphocyte proliferation was evaluated by ^{3}H-Thymidine incorporation, measured by β-radiation scintillation counting as described in the examples.

Panel B shows the effect of 100 and 1,000 IU /ml of IFN in combination with 500 U/ml of GM-CSF on the ability of DCs to induce proliferation of allogeneic lymphocytes at a stimulator to responder ratio of 1:20 (allogeneic MLR). Histograms represent ^{3}H-Thymidine incorporation evaluated by β-radiation scintillation counting.

Panel C shows IFNγ production in the supernatants from allogeneic MLRs after 5 days of co-culture. PBLs from each of four different donors were stimulated at a stimulator: responder ratio of 1:20, with allogeneic DCs generated by culturing the cells in the presence of either IFNαn/GM-CSF or IL-4/GM-CSF for 3 days. Each bar represents IFNγ concentration in the supernatant from MLR cultures of DCs with PBLs from individual allogeneic donors, as evaluated by commercial ELISA kit.

Figure 7 shows the comparative analysis of DC chemotactic response to β-chemokines. DCs were generated with different preparations of type I IFN or IL-4 in the presence of GM-CSF. $5x10^5$ DCs, generated after 3 days of treatment with GM-CSF and the indicated cytokine were resuspended in complete medium and seeded in the upper compartments of 8μm-pore size filter transwell chambers, while 0.5 μg/ml of the relevant chemokine in serum-free medium were added to the lower compartments. The lower wells of control chambers contained medium alone. Bars represent the number of cells migrated to the lower compartment, in response to chemokines, after a 2 hr incubation. Assays were performed in triplicate.

Figure 8, panel A, shows the expression at mRNA level of the chemokine MIP-3β and its receptor CCR7 in IFN-DCs as compared to IL-4-DCs. PCR products were photographed upon transillumination of 1.5% agarose electrophoresis gels stained with ethidium bromide. Panel B shows the migratory response of IL-4-DCs vs. IFN-DCs, generated with GM-CSF and different IFN preparations as indicated, elicited by MIP-3α and MIP-3β. Chemotactic assays were performed as described for fig. 7. Bars represent the number of cells migrated to the lower compartment, in response to chemokines, after a 2 hr incubation. Assays were performed in triplicate.

Panel C shows chemokine expression in IFN-DCs vs. IL-4-DCs as evaluated by RT-PCR analysis performed after 3 days of treatment of monocytes with either IFN/GM-CSF or IL4/GM-CSF. PCR products were photographed upon transillumination of 1.5% agarose electrophoresis gels stained with ethidium bromide.

Figure 9 shows the "in vitro" induction of primary immune response to HIV-1 antigens in PBLs cocultivated with autologous DCs pulsed with inactivated HIV-1.

DCs were generated by treatment of freshly isolated monocytes with different type I IFN preparations and GM-CSF or IL-4/GM-CSF for 3 days as described in the Examples. PBLs were stimulated on day 0 and restimulated on day 7 with the autologous DCs pulsed with AT-2-inactivated HIV-1 at a stimulator:responder ratio of 1:4. Control cultures were incubated with unpulsed autologous DCs. Exogenous IL-2 (25 U/ml) was added every 4 days. At day 14, the cultures were re-stimulated with DCs pulsed with AT-2 inactivated HIV-1 and, after 24 hr, ^{3}H-thymidine was added. Cells were harvested after a 18 hr incubation. Cells and supernatants from the cell cultures were tested for IFNγ production by ELISPOT analysis (panel B) and ELISA (panel C).

Panel A shows the results of lymphocyte proliferation assays to HIV antigens using DCs as APCs, as evaluated by ^{3}H-Thymidine incorporation and β-radiation scintillation counting. Black bars represent ^{3}H-Thymidine incorporation by PBLs co-cultivated with autologous DCs pulsed with inactivated HIV-1; white bars represent the ^{3}H-Thymidine incorporation by control cultures.

Panel B shows the frequency of IFNγ-producing cells in cultures of PBLs stimulated with virus-pulsed DCs, as determined by enumeration of single IFNγ-producing cells by

ELISPOT, using cells harvested at 24 hr after the 3rd stimulation with virus-pulsed DCs. Each bar represents the mean spot number of triplicates ± S.D. per 10^6 T cells. The number of HIV-reactive IFNγ-producing cells were calculated by subtraction of mean spot number of T cells induced by autologous unpulsed DCs from mean spot number of T cells induced by virus-pulsed DCs.

Panel C shows the levels of IFNγ and IL-4 production, assessed by ELISA of the supernatants of primary cultures stimulated as described above. Grey bars indicate cytokine concentration in the supernatant from PBLs co-cultured with virus-pulsed DCs, whereas white bars represent cytokine concentration in supernatant from control cultures.

Figure 10 depicts a representative experiment of "in vivo" induction of human primary response to HIV antigens in the hu-PBL-SCID mouse model (for experimental details, see examples). Panel A shows human anti-HIV-1 gp160/120 and p24 antibodies (total Ig) in the sera from individual hu-PBL-SCID mice immunized and boosted (7 days later) with $1.5x10^6$ IFN-DCs or IL-4-DCs pulsed (2 hr at 37°C) with AT-2 inactivated HIV-1 (IFN-DCs were obtained by treatment with IFNαn and GM-CSF for 3 days). Values were obtained by densitometric scanning of the corresponding bands after western blot assay. Panel B shows anti-gp41 antibody isotype characterization at days 7 and 14; bars represent the mean values obtained from three mice vaccinated with virus-pulsed IFN-DCs or IL-4-DCs. Panel C shows the "in vitro" neutralization activity against HIV of sera from immunized hu-PBL-SCID mice collected at day 21. Serial dilutions of sera from immunized hu-PBL-SCID mice were combined with 10 $TCID_{50}$ of HIV-1 SF162 strain and added to PHA activated PBMC. After 3 days, supernatants were assayed

for p24 production. Plots represent neutralizing activity of sera from individual mice immunized with the different DCs. Panel D shows the level of human IFNγ production in the peritoneum of immunized and control hu-PBL-SCID mice, as evaluated by ELISA. Fig. 11 Time course of IFNγ production by total PBL and highly purified CD8+ in vitro cultures upon stimulation with autologous inactivated HIV-pulsed IFN-DCs.

Total lymphocytes and purified CD8+ lymphocytes (>98%) were stimulated 4 times with autologous IFN-DCs pulsed with AT2-inactivated HIV-1 virions at 7 days intervals. Culture supernatants were assayed for IFNγ production 4 days after each stimulation.

Figure 12. Quantification of CD8+ T cell Response to HIV-1 antigens using IFN-gamma ELISPOT Assay

HIV-specific IFNγ producing CD8+ cells from xenochimeric mice vaccinated with IFN-DC (black bars) or IL-4-DC (open bars).

Autologous BLCLs were infected with HIV-I (SF162)(5.000 TCID50/106cells) and recombinant HIV-1 vaccinia virus vectors (MOI 3)for 48 and 12 hours respectly, washed, irradiated and used as antigen presenting cells. Unpulsed BLCL and BLCL infected with vaccinia virus vector were used as negative controls; PHA (1µg/ml) stimulus was used as positive control. PBMCs were added at $1x10^6$ per well and incubated at 37°C with 5%CO2 overnight in a final volume of 2 ml medium (RPMI 1640 supplemented with 2 mM l-glutamine and 10% heat inactivated fetal calf serum) .

After incubation with autologous BLCL, CD8+ T cells were positively selected with MACS Micro Beads (Miltenyi Biotec GmbH) and tested in an ELISPOT assay for the production of

IFN-γ (Euroclone Ltd UK). 2,5x104 CD8+ T cells, in 100µl/per well, were dispensed in a 96 well anti-gamma interferon antibody coated plate (in RPMI 1640 supplemented with 2 mM l-glutamine and 10% heat inactivated fetal calf serum); after overnight incubation and cell lysis, trapped cytokine molecules were revealed by a secondary biotinylated detection antibody and developed by incubating with streptavidin-alkaline phosphatase followed by incubating with BCIP substrate in a gel overlay. Coloured spots were enumerated on an inverted microscope at a magnification of 40. Specific IFNγ-spot forming cells were calculated by subtraction of background spots, elicited by control uninfected BLCL or by vaccinia vectors infected BLCL targets.

Figure 13 Quantification of CD8+ T cell response to the HIV-1 CTL epitope SL9 using IFN-gamma ELISPOT Assay. BLCLs were pulsed with 10^{-5}M "SL9" peptide in a volume of 50µl, washed and used as reported above. IFNγ spot forming CD8+ cells in the peritoneal cavity and spleen from xenochimeric mice vaccinated with IFN-DC (black bars) or IL-4-DC (open bars).

Figure 14 Co-cultivation assay and proviral load in vaccinated hu-PBL-SCID mice challenged with HIV-1.

Hu-PBL-SCID mice were reconstituted with human PBLs as described above and vaccinated 3 days after reconstitution by injection of IFN-DCs pulsed with inactivated HIV-1 as described. A boost injection with antigen pulsed IFN-DCs was given 7 days later and a second recall injection consisting of free purified inactivated HIV virions, was given after further 7 days. Immunized xenochimeric mice were challenged intraperitonally with 10^2 $TCID_{50}$ of SF162

seven days after a complete immunization schedule and sacrificed 7 days later.

Panel A shows viral p24 levels in supernatant from co-cultures of PHA-activated PBLs with peritoneal cells rescued from infected mice.

Panel B shows the PCR analysis for HIV-1 proviral sequences in spleens and lymph nodes from challenged xenochimeric mice.

Figures 15A - 15B - 15C Frequencies of EBV antigen-specific T cells after stimulation of PBLs with IFN-DCs or IL-4-DCs pulsed with HLA class I-restricted EBV peptides. Total PBMCs were isolated from the indicated donors. After Ficoll-Percoll separation, the $CD14^+$ monocytes were purified by immunomagnetic method, used as fresh or cryopreserved samples, and the T cell-enriched fraction was cryopreserved in aliquots. DCs were generated by culturing monocytes at 2 x 10^6 cells/ml in the presence of GM-CSF/IFN or GM-CSF/IL-4 for 3 days and then pulsed with 10 µg/ml of EBV-derived peptides, known to be CTL epitopes presented by the HLA of the selected donors. Peptide-pulsed DCs were added to autologous T cell-enriched PBLs. After 3-4 days, 10 U/ml of IL-2 were added to the cultures. T cells were restimulated with peptide-pulsed DCs, generated from cryopreserved monocytes, at 7 and 14 days after the initial co-culture. ELISPOT assays were performed after 7 days from each stimulation, in order to evaluate the number of T cells producing IFN-γ after an overnight incubation with autologous LCL (for donors FZ and FB) or peptide-pulsed T2 ($TAP^{-/-}$, HLA-A2) cells (for donor LL). Results of the ELISPOT assays performed after two rounds of stimulation of T cell-enriched PBLs with autologous peptide-pulsed IFN-DCs or IL-4-DCs are shown. Each bar represents the mean spot number of triplicates ± SD per 2 x 10^4 or 5 x 10^4 T cells. The number of peptide-reactive cells were calculated by

subtraction of mean spot number of T cells induced by autologous unpulsed monocytes from mean spot numbers of T cells induced by LCL (for donor FZ or FB) or peptide-pulsed T2 cells (for donor LL).

Figure 16. Cytotoxic activity of $CD8^+$ T cells stimulated with IFN-DCs or IL-4-DCs pulsed with HLA class I-restricted EBV peptides. $CD8^+$ T lymphocytes were immunomagnetically purified from donor FB PBLs and stimulated with unpulsed or peptide-pulsed IFN-DCs or IL-4-DCs at a stimulator to responder ratio of 1:10. Peptides used for pulsing of DCs were the same indicated in Figures 15A, 15B, 15C. Six days after the third stimulation, $CD8^+$ cells were assayed for cytotoxic activity against autologous unpulsed or peptide-pulsed LCL in standard ^{51}Cr-release assays. Specific cytotoxicity was determined according to the formula: percentage of specific lysis = 100 x [(cpm experimental release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)].

Figure 17 Sustained expansion of $CD8^+$ cells with a memory phenotype after stimulation with EBV-peptide-pulsed IFN-DCs. Dot plot FACS analysis of $CD8^+$ T lymphocytes purified from donor FB PBLs and double stained with isotype-matched control antibodies (A) or anti-CD45RA and anti-CD27 antibodies: before stimulation (B); after two rounds of stimulation with unpulsed IL-4-DCs (C) or IFN-DCs (D), or with EBV peptide-pulsed IL-4-DCs (E) or IFN-DCs (F); after three rounds of stimulation with EBV peptide-pulsed IL-4-DCs (G) or IFN-DCs (H). Peptides used for pulsing of DCs were the same indicated in Figures 15A, 15B, 15C.

Figure 18 Dot histogram analysis of the immunophenotype of IFN-DCs obtained by treating total PBMCs for three days with 1000 IU/ml or 5000 IU/ml of interferon and 500 U/ml of GM-CSF. PBMCs were obtained and treated as described in

example 11. Figure 18A relates to IFN-DCs without any maturation treatment. Figure 18B relates to IFN-DCs after maturation treatment (polyIC and anti-CD40). Mean fluorescence intensity is indicated.

Figure 19 Mixed Lymphocyte Reaction results using IFN-DCs obtained after 3 days of monocytes differentiation in presence of GM-CSF (500 IU/ml) and either native IFN-α (5000 IU/ml) or recombinant IFN-α (5000 IU/ml) after 40 hours of maturation in presence of polyIC and anti-CD40 (mDC), or without maturation (iDC). The allo-antigen presentation of these IFN-DCs was evaluated by the proliferation of allogeneic PBMC derived from an healthy donor (HD 13134-), after 5 days of co-culture between IFN-DCs (APC) and PBMC at decreasing APC/PBMC ratios. The results are expressed as mean level of 3H-dTTP incorporated into the cells due to the PBMC proliferation (cpm) for the various APC/PBMC ratios performed in triplicate.

Detailed description of the invention

Process for deriving DCs in vitro

Any mononuclear cell culture, such as purified or partially enriched CD14+ monocytes or PBMCs (peripheral blood mononuclear cells) fractions, anyway obtained by a skilled person from human or animal tissues, can be treated according to the invention.

Blood-derived highly purified $CD14^+$ monocytes, adherent PBMCs or total PBMCs, which can be collected directly from patients without any prior pharmacological treatment to mobilize DC precursors, are however particularly suitable. For subsequent clinical use, cell collection is carried out by cytapheresis or by density gradient centrifugation of concentrated leukocyte apheresis. Cells are cultivated by standard equipments, flasks and incubators suitable for clinical use.

Total PBMCs, partially enriched or highly purified monocytes are then directly cultivated in the presence of type I IFN. Monocytes can be purified by depleting contaminating lymphoid cells using positive immunoselection by anti-CD14 microbeads (MACS Cell Isolation Kits, Miltenyi Biotec, Germany). Alternatively, microbeads conjugated to a monoclonal anti-hapten antibody directed to a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA and CD56 antibodies (MACS Cell Isolation Kits, Miltenyi Biotec, Germany) are used, as recommended by manufacturer.

Any other procedures or series of procedures ensuring production of a population of monocytes, can be used as well according to the invention.

In a particularly appropriated procedure, cells are processed and cultured in "closed processors" such as a VACcell® processor (23), which include cell cultivation at 37°C in 5% CO_2 humified air in gas-permeable hydrophobic bags (21), with medium and autologous serum, in the presence of preferably 1,000 IU /ml of type I IFN and preferably 500IU/ml of GM-CSF. Serum-free media, human AB or autologous serum can be conveniently used as recognized by anyone skilled in the art to which the invention belongs.

Different types of standard media (e.g. RPMI-1630, MEM, Iscove's modified Dulbecco's Medium, Dulbecco's modified Eagle Medium) are used according to the subsequent use of DCs, whereas media suitable for treatment of human patients, such as X-VIVO 20 or AIM-V, are preferably used for culturing DCs to be employed in clinical protocols.

With regard to type I IFN suitable in the process of the invention, any type I IFN preparation can be used in the generation of IFN-DCs: natural IFNα (IFNαn) from stimulated leukocytes from healthy subjects or natural lymphoblastoid IFNα, recombinant IFNα such as IFNα2b and

IFNα2a, , consensus IFN α (CIFN), natural and recombinant IFNβ. Preferably, type I IFN is used at a concentration greater than 100 IU /ml, preferably comprised in a range of 100-10,000 IU /ml, or more preferably in a range of 400-10,000 IU /ml, or 500-2,000 IU /ml, particularly 1,000 IU /ml. With regard to the up-regulation of costimulatory molecules, the optimal enhancing effects is observed with IFN doses ranging from 500 to 1,000 IU /ml, while 100 IU /ml of IFN does not result in any significant effect. Comparable enhancing effects on DC phenotype are obtained using different preparations of type I IFN such as natural IFN-α, IFNα2b, CIFN and IFNβ.

Concentration adjustments could be necessary, following use of previously untested commercial preparations. A skilled person can in any case apply such an adjustment in function of the different IFN used and/or different culture condition used.

According to the invention, addition of IFN to the culture can be replaced by treatment with any substance capable of inducing type I IFN in culture, provided that the final concentration falls within the ranges above indicated.

Preferably, the process of the invention is carried out in the presence of a growth factor. Preferably said growth factor is GM-CSF, which is preferably used at a concentration between 250 and 1000 IU/ml.

The treatment of mononuclear cells with type I INF and optionally a growth factor is generally carried out for a maximum of three days, at the end of which, non-adherent and loosely adherent DCs are collected.

Preferably, the cells recovered between day 2 and day 3 are used directly or purified by either elutriation in a counter current centrifuge or by immunomagnetic negative selection using beads conjugated to lineage specific

antibodies. Alternatively, DCs can be conveniently cryopreserved for successive use.

In a particular embodiment of the invention, the process may include, following the derivation of DCs from mononuclear cells or from monocytes, a step of further maturation of the DCs, the maturation agent used being chosen among known maturation agents, such as a bacterial extract, poly-IC or CD40 ligand.

Object of the present invention is also a kit for the preparation of dendritic cells. This kit contains means for the reduction to practice of the process described in the present application. Those means may include: possible means for the recovery of mononuclear cells from PBMC; appropriate buffer, wash and cells conservation solutions; means for preparing a culture medium for the mononuclear cells, and complements for the culture medium, such as type I IFN and possibly GM-CSF.

A kit for deriving DCs from mononuclear cells in culture, may comprise:

- single use elements necessary for the culture and the washings of the cells, including bag(s), culture medium, buffers and connecting tubes
- a composition comprising type I IFN and compatible additives,
- possibly a composition comprising a cell growth factor and compatible additives, and
-. possibly a composition comprising antigens, or nucleic acids encoding for antigens, to which an immune response is of interest.

A skilled person can easily identify the additives suitable in the compositions reported above, among the chemically compatible additives known in the art.

A kit according to the invention may also possibly contain:

- Means for recovering and centrifuging blood to obtain a leucocyte concentrate
- Means for recovering lymphocytes and monocytes from other white cells and contaminating red cells
- Appropriate means for the conservation of the cells, means for freezing cells, including for example glycerol or Di MethylSulfoxyde in the presence of autologous or AB+ serum, or human serumalbumin.
- Means for transfection of cultured cells and means for targeting antigens to the dendritic cells of the invention.s

This kit is preferentially a single use kit. It may be, for example, a variant of the VacCell cell processor from IDM (Paris, France).

In a particular embodiment of the invention, the DCs obtained by the process described in the patent application may be loaded with antigenic peptides or proteins, with a cellular extract containing at least one antigen or with nucleic acid molecules. The cellular extract may consist of a cellular lysate or of apoptotic bodies prepared from the cells. Cells envisaged for this preparation may be lineage cells or autologous cells previously taken from the patient. Cells may be antigen loaded by pulsing with peptides, or by phagocytosis, pinocytosis, affinity binding, fusion, nucleic acid transfer or receptor mediated uptake, according to methods known by a man skilled in the art.

Type I IFNs used according to the above mentioned conditions were shown to induce a rapid differentiation of freshly isolated, preferably GM-CSF-treated human monocytes, into DCs endowed with potent functional activities both "in vitro" and "in vivo" in hu-PBL-SCID mice (24) and migration capability in response to chemotactic factors.

The comparison of DCs generated in the presence of IFN/GM-CSF with those obtained after IL-4/GM-CSF treatment revealed that type I IFN was definitively superior in inducing a rapid and stable differentiation process and in conferring a full capability to trigger a potent primary human immune response both "in vitro" and in hu-PBL-SCID mice.

DC characterization: FACS analysis, cytokine expression, chemotactic properties

A first indication of the different state of the two DC populations comes from FACS analysis revealing considerable differences in terms of membrane marker expression.

In this connection, three major types of phenotypic differences were in particular observed:

i. an early detachment of monocytes from culture plates in IFN-DCs, paralleled by rapid acquisition of high levels of CD40, CD54, CD80, CD86 and HLA-DR molecules within 3 days (Fig. 1), whereas IL-4/GM-CSF-treated monocytes required at least 6-7 days to fully acquire the immature DC phenotype;

ii. a selective expression of CD83 and CD25 (typical markers of mature DCs) in a considerable percentage of IFN-DCs (Fig. 1, Table 1); notably, in this connection CD83 expression was invariably associated with higher levels of HLA-DR and CD86; and

iii. the significant expression of the membrane antigen CD123 (IL-3-receptor a-chain) (Fig. 1), which was much more expressed in IFN-DCs than in IL-4-DCs.

A further indication came from morphological analysis of the kind of DC population (Fig. 4), that revealed that IFN-DCs rapidly acquired typical DC features within 2-3 days, with the formation of markedly oriented dendrites, as clearly detected by immunocytochemistry after CD44

staining. The polarized CD44 staining of dendrites was further typical of IFN-DCs.

Notably, upon cytokine removal, IFN/GM-CSF-treated cultures retained the DC phenotype, without adhering to the flask surface, whereas IL-4/GM-CSF-treated DCs re-acquired the macrophage characteristics and readily re-adhered to culture flasks within three days, unless stimulated to terminally differentiate.

A third indication of the mature/activated state of IFN-DCs *vs.* the immature state of IL-4-DCs came from the analysis of cytokine expression in the two DC populations, showing that IL-15 was expressed in IFN-DCs but not in IL4-DCs (Fig. 5).

A fourth indication came from the analysis of chemotactic properties. In fact, monocyte-derived IFN-DCs exhibited, with respect to IL-4-DCs, an enhanced expression of CCR5, which was associated with an enhanced migratory response to inflammatory β-chemokines (especially MIP-1β). Likewise, IFN-DCs expressed higher levels of CCR7 mRNA than IL-4-DCs along with an enhanced expression of CCR7 natural ligand, i.e. MIP-3β (Fig. 8A), consistent with the acquisition of a mature state. Notably, in this connection IFN-DCs showed potent migration response to MIP-3β, which was virtually absent in IL-4-DCs (Fig. 8B).

Of interest, the "in vitro" migration response to MIP-3β is associated with maturation, as evidenced by the CD83 up-regulation in virtually all the migrated cells, further indicating that IFN-DCs had acquired an irreversible commitment towards maturation.

The evaluation of the chemokine expression (Fig. 8C) in IFN-DCs vs. IL-4-DCs revealed other major differences, which are consistent with a differential polarization of the immune response by the two DC populations. In particular, IFN-DCs expressed high levels of IP-10 and

IL-15, while IL-4-DCs preferentially expressed MDC and TARC.

Functional analysis

The method object of the invention presented herein is useful for the culture and rapid production of DCs to be used "in vitro" and "in vivo".

The production of large quantities of clinical grade DCs with type I IFN and GM-CSF allows their use as cellular vaccine adjuvant.

DCs generated in the presence of IFN/GM-CSF according to the process of the invention showed a potent ability to take up, process and present inactivated virus to autologous T lymphocytes "in vitro", which was clearly superior to that observed using DCs cultured with IL-4/GM-CSF (Fig. 9).

On the basis of these "in vitro" results, in particular the capability of HIV-1-pulsed DCs generated in the presence of either IFN/GM-CSF or IL-4/GM-CSF to elicit a primary human immune response "in vivo", has been evaluated by using SCID mice reconstituted with autologous PBL.

Remarkably, immunization of hu-PBL-SCID mice with autologous IFN-DCs pulsed with AT-2-inactivated HIV-1 resulted in the generation of a potent primary immune response towards HIV-1 antigens (Fig. 10A), as evaluated by the detection of specific human antibodies against the whole spectrum of viral proteins (not shown). At 7 days after immunization, human antibodies proved to be mostly IgM, while HIV-1-specific IgG1 antibodies were detected at 2 weeks, suggesting a Th1-like response (Fig. 10B).

Notably, the antibodies detected in the sera of mice injected with DCs generated in the presence of IFN had a potent neutralizing activity "in vitro" against HIV-1 (Fig. 10C).

The levels of human antibodies to HIV-1 were consistently higher in hu-PBL-SCID mice injected with DCs generated in the presence of type I IFN as compared to those detected in the xenochimeras immunized with the corresponding virus-pulsed DCs developed in the presence of IL-4.

Furthermore, as shown in Example 9 below, the immunization with IFN-DCs pulsed with HIV antigens resulted in a clear cut reduction in proviral DNA load in the organs of immunized mice challenged with HIV.

In order to verify the ability of IFN-DCs to stimulate a $CD8^+$ T cell specific response, DCs were generated from monocytes of three different donors in the presence of GM-CSF/IFN and pulsed with HLA class I-restricted peptides derived from different EBV antigens and used to stimulate autologous PBLs. . As discussed in Example 10 below, INF-DCs were able to efficiently promote *in vitro* the expansion and survival of EBV-specific $CD8^+$ cells of all three donors. In order to evaluate whether the EBV-specific $CD8^+$ T lymphocytes expanded after stimulation with peptide-pulsed IFN-DCs were capable of inhibiting lymphomagenesis in the hu-PBL-SCID chimeric model, SCID mice were reconstituted with PBMCs from one of the donors, previously tested for the ability of forming lymphomas after PBMCs injection into SCID mice. Vaccination of the reconstituted animals with autologous peptide-pulsed IFN-DCs caused a highly significant prolongation of survival time as compared to what observed for unvaccinated SCID mice and for mice vaccinated with unpulsed IFN-DCs (Table 2). Overall, these results indicate that IFN-DCs are efficient in stimulating the expansion of effector $CD8^+$ T lymphocytes.

The results obtained also indicate that IFN-DCS can efficaciously be used for the prevention and treatment of

pathologies associated with the presence of antigens in the body. Preferably said pathologies are infectious or neoplastic diseases. The DCs of the invention are particularly suitable for the prevention and treatment of lymphomas, of viral infections such as HIV, HBV or HCV infection and virally induced neoplastic diseases such as those induced by Epstein Barr virus.

Therefore, the present invention also refers to vaccines and pharmaceutical compositions comprising, as an adjuvant, the DCs of the invention, together with at least one immunogen and a pharmaceutically acceptable carrier vehicle or an auxiliary agent.

A further object of the present invention are pharmaceutical compositions and vaccines comprising as an active principle the dendritic cells of the invention, preferably loaded with one or more antigens, together with a pharmaceutically acceptable carrier, vehicle or auxiliary agent.

The antigen(s) may be loaded on the Dcs of the invention by pulsing for 1-2 hours at 37°C with peptides (in the range of 10-200 µg/ml), selected on the basis of the patient HLA haplotype and the type of response to be elicited.Alternatively, the IFN-DCs of the invention can be pulsed with whole proteins or protein complexes.

In the case of malignancies exhibiting unknown tumor-associated antigens, IFN-DCs can be pulsed with tumor RNA complexed to cationic liposomes or with whole tumor cell lysates. Moreover, IFN-DCs can be induced to take up antigens by engulfing apoptotic or necrotic tumor cells or by exposure to cell lysates. In such cases, incubation time can be appropriately prolonged up to 4-5 hours. In fact, IFN-DCs were demonstrated to be able to phagocytose cell lysates, as well as fragments from

apoptotic or necrotic tumor and virally infected cells "in vitro".

IFN-DCs can also efficiently internalize viral particles, bacteria and yeasts, permitting the targeting of multiple epitopes or complex antigens to DCs via inactivated or genetically-modified microorganisms. Moreover, even engineered DNA and RNA can be directly internalized, to deliver antigen-coding sequences to IFN-DCs.

In some cases, IFN-DCs do not need any pulse or tumour antigen administration before utilization, as in the case of IFN-DCs from chronic myelogenous leukemia (CML) patients, whose $CD14^+$ monocytes already express the BCR-ABL fusion gene or other putative tumor antigens.

Possible routes of administration of antigen-loaded IFN-DCs are any route used for administering vaccine and include, whatever this antigen is, intranodal, subcutaneous, intravenous, intraperitoneal, intramuscular, transdermal or intradermal injections, including intratumoral injection. An alternative modality of administration includes the slow i.v. infusion even with auxiliary external infusion pumps.

An additional modality of administering IFN-DCs can involve their direct injection within primary tumor or viral lesions, metastases or regional draining lymph node, even without prior incubation with specific antigens, which are locally acquired by IFN-DCs soon after injection. Administration modality and time schedule are designed and adjusted according to the age and weight of the patient, the disease and its severity as well as the response rate. Thus, 2×10^6 to 5×10^7 IFN-DCs can be infused once or at weekly/monthly time intervals according to the procedures described above.

IFN-DCs loaded with antigens can also be used for the "ex vivo" expansion of T cells, e.g. $CD4^+$ and/or $CD8^+$ or

both, to be re-infused in patients. Such immune intervention can be useful in therapy of humans having immune disorders or deterioration, as in the course of persistent infections or neoplastic diseases.

EXAMPLES

Example 1. Derivation of DCs from monocytes and characterization of immunophenotype and morphology thereof

Derivation of DCs from monocytes

Peripheral blood mononuclear cells were obtained from heparinized blood of normal donors by Ficoll density gradient centrifugation (Seromed). Monocytes were obtained either by 2 hr adhesion in 25-75 cm^2 flasks (Costar, Cambridge, MA) or by standard Percoll density gradient centrifugation.

Monocytes were further enriched by depleting contaminating cells using negative immunoselection by microbeads conjugated to a monoclonal anti-hapten antibody directed to a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA and CD56 antibodies (MACS Cell Isolation Kits, Miltenyi Biotec, Germany). After these procedures, the resulting cell population was represented by >95% $CD14^+$ monocytes, as assessed by flow cytometry.

Blood derived monocytes were plated at the concentration of 1-2 x 10^6 cells/ml in RPMI 1640 (Gibco BRL, Gaithesburg, MD) supplemented with 10% FCS. GM-CSF (500 U/ml) was added in combination with the following cytokines: IL-4 (500 U/ml) (R & D Systems, Minneapolis, MN) and natural IFNα (1,000 IU /ml): IFNαn (Alfaferone Alfa-Wassermann).

All the IFN preparations used were shown to be free of any detectable LPS contamination. After 3 or 6 days of culture, non-adherent and loosely adherent cells were collected and used for subsequent analysis.

The experiments were carried out in order to compare type I IFN + GM-CSF treatment with IL-4 + GM-CSF treatment, currently used for obtaining immature DCs from monocytes in 6-7 days.

It has been observed in this connection that in response to IFN/GM-CSF treatment, adherent monocytes rapidly became floating non-adherent cells within 3 days. The loss of adherence was associated with cellular aggregation and large cell clusters were detected in the IFN/GM-CSF-treated cultures, while a large part of IL-4/GM-CSF-treated cells were still firmly adherent to the plastic surface. DCs so obtained have been therefore further characterized immunophenotipically and morphologically.

DC immunophenotypical characterization

Cells were washed and resuspended in PBS containing 1% human serum and incubated with a series of fluorochrome-conjugated mAbs to human antigens for 30 min at 4°C. The following mAbs were used for immunofluorescent staining: anti-CD14, -CD25, -CD54, -CD80 and -HLA-DR (Becton Dickinson, San Jose CA), -CD1a, -CD23, -CD40, -CD83 and -CD86 (Pharmingen, San Diego CA). Cells were analysed by flow cytometry. Data were collected and analysed by using a FACSort (Becton Dickinson) flow cytometer; data analysis was performed by CellQuest software (Becton Dickinson). DCs were electronically gated according to light scatter properties in order to exclude cell debris and contaminating lymphocytes.

After 3 days of culture, cells treated with either IFN/GM-CSF or IL-4/ GM-CSF were analyzed for the expression of surface markers associated with DC differentiation as well as of the monocytic marker CD14. Monocytes were purified by standard Ficoll and 46% Percoll density gradient centrifugations followed by immunomagnetic sorting. Fig. 1 illustrates the expression of selected

markers upon treatment of monocytes with 1,000 IU /ml of IFNαn and 500 U/ml of GM-CSF (panel A) as compared to treatment with and 500 U/ml of IL-4 and 500U /ml of GM-CSF (panel B). The up-regulation of costimulatory molecules (CD80, CD86 and CD40) was consistently higher in IFN-DCs than in IL-4-DCs as early as 3 days after cytokine treatment. Comparable enhancing effects on DC phenotype were observed using different type I IFNs (i.e., IFNαn, IFNα2b, CIFN and IFNβ) added in conjunction with GM-CSF to blood-derived monocytes for 3 days of culture. To this regard, Fig. 2 shows the comparison of the representative FACS profiles obtained at 3 days of cytokine treatment, wherein monocyte fraction was enriched by standard Ficoll density gradient centrifugation and subsequent centrifugation on 46% Percoll density gradient, and the different IFN preparations were used at the same concentration of 1,000 IU /ml.

Results of this comparison indicate that all type I IFN preparations are suitable for generating DCs.

In this connection, the following Table 1 summarizes the immunophenotypic features of DCs obtained from blood monocytes treated with 500 U/ml GM-CSF and 1,000 IU /ml of either IFNαn, IFNβ, or CIFN as compared to IL-4-DCs after three days of cytokine treatment. Freshly isolated monocytes were partially purified by Ficoll and Percoll density centrifugation and treated as described above. DCs were analyzed by flow cytometry, gating DCs according to light scatter properties. Data were acquired and analyzed by using a FACSort instrument and "Cell Quest" software (Becton Dickinson). Values represent the mean ± S.D. of nine experiments (three different experiments for each different type I IFN preparation). Values are obtained by dot histogram analysis of antigen expression and represent

the mean percentage of positive cells for a given surface antigen and its Mean Fluorescence Intensity (MFI).

Table 1. Phenotype of IFN- and IL4-DCs after 3 days of cytokine treatment

	IFN-DCs		IL-4-DCs	
Marker	Percentage ± S.D.	MFI ± S.D.	Percentage ± S.D.	MFI ± S.D.
CD40	96±11	87±15	63±11	26±15
CD80	91±10	175±83	70±12	34±7
CD86	79±20	254±105	70±7	81±31
CD83	25±14	52±6	1±3	43±5
CD25	23±12	50±10	1±0.5	33±3
HLA-DR	96±3	2060±467	97±2	1121±263
CD54	95±4	641±113	94±3	239±69
CD14	36±18	60±12	13±8	53±15
CD1a	41±17	105±21	72±17	284±32

Notably, monocytes treated with IFN showed not only a marked up-regulation of costimulatory molecules and HLA-DR antigen, but also a clear-cut induction of the expression of the CD83 (15-40% of positive cells) and CD25 antigens, both considered as markers of mature/activated DCs. On the contrary, CD83 was expressed only by a strict minority of IL-4/GM-CSF-cultured DCs (1-4%).

The effects of different doses of type I IFN and in particular doses of 1,000 IU /ml, 500 IU /ml and 100 IU /ml have been therefore evaluated. Freshly isolated monocytes were isolated, cultured with cytokines and analyzed for antigen expression on day 3, to this purpose.

The relevant dose-response results shown in Fig. 3, indicated that the optimal type I IFN concentration for the upregulation of costimulatory molecules is within the range of 500-1,000 IU /ml, while 100 IU /ml of IFN did not result in any significant effect.

On the whole, these results underline that a 3-day exposure of freshly isolated monocytes to type I IFN/GM-CSF instead of IL-4/GM-CSF results in the generation of a characteristic type of partially mature DCs, as evidenced in particular by the significant expression of CD83 and CD25. These data show that IFN treatment not only induced an upregulation of costimulatory molecules, but also promoted the appearance of partially activated $CD83^+$ DCs.

The irreversible commitment of IFN-DCs to undergo an advanced maturation process was suggested by the finding that, upon cytokine removal, these cells retained a DC phenotype without adhering to the plastic surface, whereas IL-4-DCs re-acquired the macrophage features and readily re-adhered to culture plates within three days, unless preventively stimulated to terminally differentiate by LPS.

DC morphological characterization

In order to further detect potentially relevant differences between IFN-DCs and IL-4-DCs, immunocytochemical analysis was performed by using CD44 antibodies, since preliminary experiments had revealed that this protein was specifically expressed on dendrites and its staining clearly outlined these structures.

IFN- or IL4-DCs obtained after a 3 day-cytokine-treatment were spun onto glass slides (Shandon, Cheshire, UK) at the concentration of 10^4 cells/ml, fixed with ethanol (70%) 10 min at + 4 °C and stained by immunocytochemistry for CD44 (Dako, Denmark) using the peroxidase-anti-peroxidase (PAP/AEC) (Dako, Denmark) method. Cells were counterstained with Mayer's haematoxilyn.

Clear-cut differences were observed in comparing IFN-DCs and IL-4-DCs at different culture times. In particular, a remarkable higher number of CD44 stained dendrites was observed in IFN-DCs as compared to IL-4-DCs (Fig. 4). The

dendrites of IFN-DCs were mostly thin and long, reaching 21-30 μm of length (3-4 times the diameter of the cell body) and unidirectionally oriented (panel A). Notably, the CD44 staining is typically localized on dendrites nicely outlining them.

On the contrary, the typical $CD44^+$ stained morphology of IL-4-DCs (panel B) was that of larger cells with squat and short dendrites that highly resemble ruffles of different size.

In general, IL-4-DCs did not show the unidirectional orientation of dendrites typical of IFN-DCs. On the whole, these results were highly consistent with those obtained by Scanning Electron Microscopy and suggested that morphologic and phenotypic features characteristic of the blood DCs were generated after 2-3 days of treatment with type I IFN.

Example 2: Production of cytokines by IFN-DCs and IL4-DCs

DCs produce a series of cytokines implicated in the initiation of the immune response especially when activated by mutual interaction with T cells or by encounter with viral pathogens and bacterial products. Thus, it was of interest to evaluate whether IFN/GM-CSF treated DCs exhibited any specific pattern of cytokine expression as compared to cells cultured in the presence of IL-4/GM-CSF. To this end a comparative RT-PCR analysis has been carried out.

Total RNA from DCs was extracted by RNAzol B and processed as previously described (24).

Transcripts were detected by amplifying the retro-transcribed RNA with specific primer pairs for:

- IL-1 sense CTTCATCTTTGAAGAAGAACCTATCTTCTT, antisense AATTTTTGGGATCTACACTCTCCAGCTGTA),

- TNFα sense ATGAGCACTGAAAGCATGATCCGG, antisense GCAATGATCCCAAAGTAGACCTGCCC),

- IL-12 p40 (sense CCAAGAACTTGCAGCTGAAGA, antisense TGGGTCTATTCCGTTGTGTC),

- IL-15 (sense CTCGTCTAGAGCCAACTGGGTGAATGTAATAAG, antisense TACTTACTCGAGGAATCAATTGCAATCAAGAAGTG)

- IL-18 (sense TCTGACTGTAGAGATAATGC, antisense GAACAGTGAACATTATAGATC);

GAPDH RT-PCR was run in parallel to normalize the levels of human RNA in all the samples. All RT-PCR products were in the linear range of amplification.

The relevant results, reported in Fig. 5A, showed that IFN-DCs expressed high levels of mRNA for IL-1β. Notably, induction of IL-15 expression was selectively detected in cultures treated with IFN/GM-CSF. As IL-15 expression is tightly regulated at the translational level, it was of interest to determine whether detectable levels of the cytokine could be revealed in the supernatants of IFN-treated cultures. Secretion of IL-15 in the supernatant of DCs differentiated in the presence of various type I IFN preparations and GM-CSF as compared to IL-4/GM-CSF treatment for 3 days is reported in Fig. 5B which shows that remarkable levels of IL-15 were secreted in response to the IFN/GM-CSF treatment.

Example3. Allogeneic stimulatory capacity of IFN-DCs

Enhanced allostimulatory properties of DCs generated in the presence of IFN/GM-CSF.

A series of functional experiments has been carried out for comparing the ability of DCs generated from monocytes in the presence of IFN/GM-CSF or IL-4/GM-CSF to stimulate proliferation and IFNγ production by allogeneic PBLs in MLR assays.

Monocyte-depleted PBLs were seeded into 96 wells plates (Costar, Cambridge, MA) at 10^5 cells/well. Purified allogeneic DCs (5 x 10^3) were added to each well in

triplicate. After 5 days, 1 μCi of methyl-^{3}H-Thymidine (Amersham) was added to each well and incubation was continued for additional 18 hr. Cells were finally collected by a Mach II Mcell (Tomtec) harvester and thymidine uptake was quantitated by liquid scintillation counting on 1205 Betaplate (Pharmacia).

As illustrated in Fig. 6A, wherein are reported the results of the comparative MLR assays in the presence of various preparations of type I IFN and GM-CSF or IL-4 /GM-CSF, IFN-DCs proved to be superior in inducing the proliferation of allogeneic PBLs as compared to IL-4-DCs, as revealed by ^{3}H-thymidine incorporation assay.

Notably, DCs generated in the presence of 100 IU /ml IFN elicited a poor proliferative response, as showed in fig. 6B, wherein the effects of the different concentrations of IFN in combination with 500 U/ml of GM-CSF on the ability of DCs to induce proliferation of allogeneic lymphocytes are reported.

This was not unexpected on the basis of the results reported above, since DCs generated with 100 IU /ml of IFN exhibited very low levels of co-stimulatory molecules, as determined by flow cytometric analysis (Fig. 3).

A specific feature of MLRs generated with IFN-DCs was the considerable IFNγ production, which was definitely higher than that found in the corresponding co-cultures using DCs generated with IL-4 (Fig. 6C), suggesting a prominent capability of IFN/GM-CSF-DCs to promote a Th1 response.

Example4. Analysis of the migratory response to chemokines

The migration and function of DCs is strictly regulated by their response to chemokines as well as by the expression of DC-derived chemokines, whose production can markedly shape DC functional activities. The attitude to

migrate in response to chemotactic stimuli was analyzed in IFN-DCs and IL-4-DCS, together with the expression of chemokines/chemokine receptors in both DC populations.

a. Response to β-chemokines

Chemotactic response to inflammatory β-chemokines was studied by measuring the migration capability of DCs using a two compartment systems with chemokine containing medium (Fig.7) Cell migration was performed in 24-well Transwell cell culture chambers (Costar). In brief, 5 x 10^5 cells cultured in complete medium with IFN/GM-CSF or IL4/GM-CSF for 3 days were resuspended in complete medium and seeded in the upper compartment of 8 µm-pore size filter Transwell chambers.

RANTES, MIP1α, MIP1β (500ng/ml) (R&D System), were diluted in serum-free medium and added to the lower compartment, while the lower wells of control chamber contained medium alone. After 2 hr incubation at 37°C, the cells migrated through the 8µm-pore size polycarbonate filters in the lower compartment were collected and counted. Each assay was performed in triplicate.

Of interest, the generation of DCs with type I IFN and GM-CSF in 3 days was associated with a stronger chemotactic response to the β-chemokine RANTES, MIP-1α and especially to MIP-1β, as compared to DCs generated with IL-4 and GM-CSF, suggesting an intrinsic attitude of IFN-DCs to promptly respond to inflammatory chemokines.

b. IFN-DCs over-express CCR7 and exhibit an enhanced capacity to migrate in response to Mip-3β.

Mature DCs have been reported to respond to MIP-3β/ELC and 6Ckine/SLC as a consequence of an up-regulation of their receptor (CCR7). Of interest, recent studies in knock-out mice for CCR7 have shown the crucial importance of the CCR7/MIP-3β interaction for the generation of a

primary immune response (25). Thus, we evaluated the expression of CCR7 in IFN-DCs as compared to IL-4-DCs. Transcripts were detected by amplifying the retro-transcribed RNA with specific primer pairs for:

- hCCR7 (sense TCCTTCTCATCAGCAAGCTGTC, antisense GAGGCAGCCCAGGTCCTTGAAG);
- hMIP3β (sense CACCCTCCATGGCCCTGCTACT antisense TAACTGCTGCGGCGCTTCATCT);

The samples were amplified for 25-35 cycles at the following conditions: 94°C 40'', 62°C 40'', 72°C 40''. To amplify hMIP-3β mRNA the annealing temperature was 58°C. α-actin RT-PCR was run in parallel to normalize the levels of human RNA in all the samples. All RT-PCR products were in the linear range of amplification. RT-PCR analysis revealed that IFN-DCs expressed higher levels of CCR7 mRNA as compared to IL-4-DCs, as shown in figure 8 (panel A), wherein the expression at mRNA level of the chemokine MIP-3β and its receptor CCR7 in IFN-DCs vs. IL-4-DCs is compared.

Of interest, when both types of DCs were tested for their capacity to migrate in response to the natural ligand of CCR7, a marked chemotactic response to MIP-3β was specifically observed for IFN-DCs. See in this connection panel B of figure 8 wherein the migratory response of IL-4-DCs vs. IFN-DCs (generated with GM-CSF and different type I IFN preparations as indicated), in response to Mip-3α and Mip-3β is compared. Thus, IFN-DCs were found to express CCR7 and to respond to its natural ligand Mip-3β very efficiently confirming that IFN-DCs, at least in part, exhibit features of mature DCs.

In another set of studies, mRNA from DCs was extracted by RNAzol B and processed as previously described to detect the expression of a set of chemokines. The following primer sets were used:

- DC-CK1 (sense ACAAAGAGCTCTGCTGCCTC, antisense CCCACTTCTTATTGGGGTCA);
- TARC (sense CCTCCTCCTGGGGGCTTCTCTG, antisense GACTTTAATCTGGGCCCTTTGTGC);
- IP-10 (sense TGATTTGCTGCCTTATCTTTCTGA - antisense CAGCCTCTGTGTGGTCCATCCTTG);
- MDC (sense CAGCCTGACAAATCACAGTG - antisense CTGGATGACACTGAGCTGG).

As shown in Fig. 8C, wherein RT-PCR analysis performed after 3 day treatment of monocytes with either IFN/GM-CSF or IL-4/GM-CSF is reported, the mRNA for DC-CK1, a chemokine specifically expressed by human DCs at high levels, was strongly expressed in IFN-DCs. Moreover, IP-10 mRNAs was expressed at higher levels in IFN-DCs with respect to IL-4-DCs, while MDC and TARC expression was up-regulated in IL-4-DCs.

Example 5: Primary response to HIV antigens elicited by IFN-DCs "in vitro": comparison with the activity of DCs generated in the presence of IL-4/GM-CSF

The ability of DCs generated in the presence of either IFN/GM-CSF or IL-4/GM-CSF to initiate a primary response in autologous PBLs was evaluated by using inactivated HIV-1 as an immunogen. To inactivate HIV, a recently described procedure (22) has been adopted, consisting in the use of 2,2'-dithiodipyridine (aldrithiol-2; AT-2), which inactivates HIV by selectively disrupting the p7 nucleocapsid (NC) protein, leaving intact the conformation and fusogenic activity of the gp120 HIV-1 protein (26).

Autologous PBLs were stimulated with DCs pulsed with AT-2-inactivated HIV-1. HIV-1 SF162 strain was inactivated by AT-2 and stored at -140°C until use. PBLs ($4x10^6$) were stimulated with 1 x 10^6 autologous DCs generated by treatment with either IFN/GM-CSF or IL-4/GM-CSF for 3 days

and pulsed with AT-2-inactivated HIV-1 (40 ng of p24) for 2 hr at 37°C. Control cultures were incubated with unpulsed autologous DCs. PBLs were restimulated 7 days later with unpulsed or inactivated virus-pulsed DCs. Exogenous IL-2 (25 U/ml) was added every 4 days. At day 14, Proliferation assays were performed as follows: $5x10^3$ unpulsed or inactivated virus-pulsed DCs were added to 10^5 autologous PBLs into triplicate wells. After 6 days, 1 µCi of methyl-^{3}H-Thymidine was added to each well and incubation was continued for additional 18 hrs. Cells were collected and thymidine uptake was quantitated as described in *Example 4*.

Cells and supernatants from the cell cultures were also tested respectively for IFNγ production by ELISPOT analysis and ELISA.

Virus-pulsed IFN-DCs not only proved to be better stimulators of ^{3}H-thymidine uptake by autologous PBLs than IL-4-DCs, but also induced a stronger Th1-oriented response. In Fig. 9 (panel A) the results of lymphocyte proliferation assays to HIV antigens using DCs as APCs are reported. The frequency of IFNγ-producing cells (assessed by ELISPOT) and the levels of IL-4 and IFNγ production (measured by ELISA) in the primary cultures stimulated as described above are reported respectively on panel B and C of the same figure 9.

The evaluation of IFNγ-producing cells was performed by ELISPOT assay (Euroclone U.K.) according to the manufacturer's instructions. Briefly, 96-well plastic plates (Maxisorp Nunc) were coated with capture anti-IFNγ antibodies and blocked with 2% BSA. Ten-fold dilutions (from 10^5 to 10^2) of PBLs from primary cultures were restimulated overnight with DCs pulsed with inactivated HIV-1, added to triplicate wells and incubated for 18 hr. After cell removal, plates were incubated with an anti-IFNγ detection biotinylated antibody and streptavidin-conjugated

alkaline phosphatase. Then, substrate solution was added and the frequency of IFNγ-producing cells was evaluated by enumerating single spots on an inverted microscope.

The ELISPOT analysis showed a higher number of IFNγ-producing cells in primary cultures stimulated with DCs generated with different preparations of type I IFN + GM-CSF as compared to cultures stimulated with IL-4-DCs, as shown in panel B of figure 9. These results were consistent with the secretion of higher levels of IFNγ in the supernatants of IFN-DCs, as shown in panel C of figure 9, wherein the levels of IL-4 and IFNγ production measured by ELISA in the supernatants of primary cultures stimulated as described above are reported. Notably, little or no secretion of IL-4 was detected in cultures stimulated with virus-pulsed IFN-DCs, while considerable amounts of this cytokine were found in the supernatants of cultures exposed to virus-pulsed IL-4-DCs (Fig. 9C).

Example 6: Primary antibody response to HIV antigens elicited by IFN-DCs in the hu-PBL-SCID mouse model: comparison with the activity of DCs generated in the presence of IL-4/GM-CSF

The evaluation of the effects of IFN-DCs on the "in vivo" primary immunization and antibody response in the model of SCID mice reconstituted with human PBLs (27), was carried out. In fact, recent data have suggested that a human primary immune response can be generated in hu-PBL-SCID mice, especially when the chimeras are injected with antigen pulsed DCs (24 , 28).

Four-week-old CB17 scid/scid female mice (Harlan, Nossan, Italy) were kept under specific pathogen-free conditions. SCID mice were housed in microisolator cages and all food, water and bedding were autoclaved prior to use. Hu-PBLs were obtained from the peripheral blood of healthy donors. All donors were screened for HIV-1 and

hepatitis viruses prior to donation. The hu-PBLs were obtained by Ficoll-Paque density gradient centrifugation. Twenty million cells were resuspended in 0.5 ml of RPMI 1640 medium and injected i.p. into the recipient mice. Mice were injected i.p. with 2 x 10^6 autologous DCs, pulsed for 2 hr at 37°C with AT-2 inactivated HIV-1 (100 ng of p24 per immunization dose). Seven days later, mice were given a boost dose of AT-2 inactivated HIV-pulsed DCs. At day 7 and 14, sera from hu-PBL-SCID mice were assayed for the presence of human anti-HIV antibodies.

The total spectrum of human antibodies against HIV-1 proteins was evaluated by performing Western Blot analysis with pooled sera from hu-PBL-SCID mice injected with virus-pulsed DCs. Sera from hu-PBL-SCID mice injected with HIV-1-pulsed DCs were assayed by Western blot (Cambridge Biotech HIV western blot Kit, Rockville MD). Briefly, nitrocellulose strips were incubated overnight with individual mouse serum samples (diluted 1:20) or with a human positive control serum (diluted 1:1,000). Visualization of the human Igs specifically bound to HIV-1 proteins was obtained by incubation with substrate chromogen after the addition of biotin-conjugated goat anti-human IgG and streptavitin-conjugated horseradish peroxidase. Western blot strips were examined by densitometry using the Quantity One 4.2.1 software (Bio Rad) to detect the intensity of serum antibody reactivity towards the HIV-1 gp120/160 and p24 antigens. The mean values detected in the sera from 3 control non-immunized Hu-PBL-SCID mice were used as cut-off to determine the specific antibody reactivity in the serum from immunized chimeras.

An ELISA system was utilized to quantitate human total Igs, IgM, IgG1 and IgG4 immunoglobulins in the sera of the chimeras by using anti-human total Ig and anti IgM (Cappel-

Cooper Biomedical, West Chester, P. A. and anti IgG1 or anti IgG4 (Pharmingen). All ELISAs were performed in duplicate and laboratory standards were included on each plate. Sera from non-reconstituted SCID mice were used as negative controls of all the ELISA determinations. ELISA for detection of specific anti-HIV antibodies was performed using a specific peptide (i.e., ERYLKDQQLLGIWGCSGKLIC) corresponding to amino acids 591 to 611 of the HIV-1 gp41 protein. Synthetic peptides were immobilised on Dynatec (Dynal, Oslo, Sweden) microtitre plates by an overnight incubation at 4° C. Serially diluted mouse sera were added and incubated for 90 min at room temperature. Finally, binding was revealed by reading A_{490} values after incubation with substrate chromogen. Values represent mean adsorbance value of each individual serum tested in duplicate. The cut-off value was calculated as mean adsorbance value of all the control sera plus 0.100 A. Sera showing A_{490} values higher than this threshold were considered positive for anti-HIV antibodies.

Hu-PBL-SCID mice immunized with DCs generated in the presence of IFN/GM-CSF showed higher levels of anti-HIV antibodies directed to gp160/120 and p24 antigens, as compared to the xenochimeras injected with DCs obtained after IL-4/GM-CSF treatment. Fig. 10 (panel A) shows, in particular, the levels of human anti-HIV-1 gp160/120 and p24 antibodies (total Ig) detected in the sera from individual hu-PBL-SCID mice immunized and boosted (7 days later) with 1.5 x 10^6 IFN-DCs or IL-4-DCs, both pulsed (2 hr at 37°C) with AT-2 inactivated HIV-1 (DCs were obtained by treatment with IFNαn and GM-CSF for 3 days). Values were obtained by densitometric scanning of the corresponding bands after western blot assay.

ELISA studies revealed the presence of high levels of anti-gp41 antibodies in hu-PBL-SCID mice immunized with

HIV-1-pulsed IFN-DCs, as shown in panel B of figure 10, wherein anti-gp41 antibody isotype characterization at days 7 and 14, is reported. In this connection moreover, at day 7, anti-HIV-1 antibodies were shown to belong mainly to the IgM isotype (Fig. 10B) whereas, at day 14, antibodies belonging to the IgG1 isotype were detected especially in mice immunized with IFN/GM-CSF cultured DCs, revealing isotype switching upon antigen boost and suggesting a stronger Th1 biased response (Fig. 10B).

Remarkably, sera from hu-PBL-SCID mice immunized with virus-pulsed IFN-DCs were capable of recognizing virtually all the HIV-1 proteins detectable by Western blot analysis using a human positive control serum, as shown in panel C of figure 10, wherein the "in vitro" neutralization activity against HIV of sera from immunized hu-PBL-SCID mice collected at day 21 is reported.

Serial dilutions of sera from immunized hu-PBL-SCID mice were combined with 10 $TCID_{50}$ of HIV-1 SF162 strain and added to PHA activated PBMC. After 3 days, supernatants were assayed for p24 production. Notably, sera from xenochimeras immunized with IFN-DCs and exhibiting high levels of anti HIV-1 antibodies effectively neutralized HIV-1 infection of activated human PBL "in vitro".

ELISA test was performed to titrate human IFNγ in peritoneal washings from vaccinated and control reconstituted hu-PBL-SCID mice. Significant amounts of IFNγ were selectively detected in vaccinated xenochimeras, with higher levels of this cytokine in peritoneal fluids from IFN-DC-vaccinated mice as compared to mice vaccinated with IL-4-DCs (fig.10D).

Example 7. Time course analysis of IFNγ production by total PBL and highly purified CD8+ in vitro cultures upon

stimulation with autologous IFN-DCs pulsed with inactivated HIV-1.

Total PBLs and immunomagnetically purified (MACS Cell Isolation Kits, Miltenyi Biotec, Germany) CD8+ lymphocytes (>98%) were stimulated four times with autologous IFN-DCs pulsed with AT2-inactivated HIV-1 virions at 7 days intervals. Culture supernatants were assayed for IFNγ production 4 days after each stimulation. Repeated stimulation with virus-pulsed IFN-DC, even in absence of CD4+ helper T cells, led to a marked IFNγ production, which started soon after the first stimulation, with IFNγ concentrations around 100pg/ml, and augmented with subsequent recall stimulations reaching a concentrations up to 2ng/ml (fig. 11). Interestingly, IFNγ production in culture supernatant was paralleled by the virtual absence of IL-4 (data not shown).

Example 8 Generation of CD8+ effector lymphocytes showing reactivity toward HIV-1 antigens and a conserved CTL epitope.

Virus-specific CD8+ lymphocytes retain a range of antiviral activities, including killing of infected cells and the ability to produce cytokines and chemokines. IFNγ is a key antiviral cytokine produced by effector CD8+ T cells at the site of virus infection, while β-chemokines suppress HIV infection by inhibiting viral entry into target cells.

We evaluated the "in vivo" elicitation of virus-specific IFNγ-producing CD8+ T cells upon vaccination of hu-PBL-SCID mice with autologous AT2-HIV-pulsed DCs. We rescued human

CD8+ T cells from mouse spleens or peritoneal cavity and evaluated CTL effectors by IFNγ Elispot assay, a reliable method for evaluating CD8 response and currently applied to map CTL epitopes. Although the cells responding to HIV in this assays are referred to as CTL, they represent IFNγ-secreting cells.

We rescued human cells from hu-PBL-SCID mice and tested them on autologous "B-lymphoblastoid cell line" (BLCL) targets, infected either with HIV-1 SF162 strain ($5x10^3$ $TCID_{50}/10^6$ cells) or recombinant vaccinia virus vectors encoding gag and pol antigens from the IIIB strain of HIV-1. Autologous BLCLs were infected with HIV-I (SF162) and recombinant HIV-1 vaccinia virus vectors for 48 and 12 hours respectively, washed, irradiated and used as antigen presenting cells. Unpulsed BLCL and BLCL infected with vaccinia virus vector were used as negative controls; PHA (1µg/ml) stimulus was used as positive control. PBMCs were added at $1x10^6$ per well and incubated at 37°C overnight in a final volume of 2 ml of medium (RPMI 1640 supplemented with 2 mM l-glutamine and 10% heat inactivated fetal calf serum). After incubation with autologous BLCL, CD8+ T cells were positively selected with MACS Micro Beads (Miltenyi Biotec GmbH) and tested in an ELISPOT assay for the production of IFN-γ (Euroclone Ltd UK). $2,5x10^4$ CD8+ T cells in 100 µl /per well, were dispensed in a 96 well anti-gamma interferon antibody coated plate, and after overnight incubation and cell lysis, trapped cytokine molecules were revealed by a secondary biotinylated detection antibody and developed by incubating with streptavidin-alkaline phosphatase followed by incubating with BCIP substrate in a gel overlay. Coloured spots were enumerated on an inverted

microscope at a magnification of 40.Specific IFNγ-spot forming cells were calculated by subtraction of background spots, elicited by control uninfected BLCL or by vaccinia vector infected BLCL targets.

As shown in fig. 12, vaccination of hu-PBL-SCID mice obtained with PBLs from different donors resulted in a CD8 response toward HIV-1 antigens. Although a certain variability could be observed between the different donors, AT2-HIV-pulsed IFN-DCs potently elicited CD8 response against the homologous viral strain SF162 as well as against gag and pol proteins from the HIV-1 IIIB strain.

We also analyzed the response toward the HLA-A*0201 restricted CTL epitope SL9 (SLYNTVATL) in vaccinated xenochimeras. This immunodominant HIV-gag p17-derived epitope is conserved and elicits strong CTL response which is generally maintained in some individuals even in the presence of strong selective pressure to viral escape. The data reported here show that vaccination of a HLA-A*0201 individual with DCs pulsed with the whole AT2-inactivated HIV virions is capable of eliciting a strong response "in vivo" toward the conserved SL9 epitope (fig.13).

Example 9. Protection of hu-PBL-SCID mice vaccinated with virus-pulsed IFN-DCs from HIV-1 infection

A group of Hu-PBL-SCID mice were challenged intraperitonally with 10^2 $TCID_{50}$ of SF162 seven days after a complete immunization schedule. Two weeks later, the xenochimeric mice were sacrificed, and the level of protection from viral challenge was evaluated. PCR analysis for viral gag sequence in spleen and lymph nodes of infected xenochimeras demonstrated a clear cut reduction in proviral DNA load in the organs of immunized mice, together with a reduced capacity to rescue infectious virus by

cocultivation of peritoneal cells with PHA-activated T lymphocytes and p24 antigen detection in culture supernatants by ELISA (Dupont, Brussels, Belgium). (fig.14).

Example 10 IFN-DCs pulsed with Epstein-Barr virus (EBV) peptides as stimulators of a $CD8^+$ T cell specific response.

We evaluated the capability of IFN-DCs vs. IL-4-DCs to stimulate an EBV-specific CD8+ T cell response, after pulsing with HLA class I-restricted peptides derived from different EBV antigens. Three donors were used for these studies: LL (HLA-A2); FZ (HLA-A3, -B35); FB (HLA-A11, -B27). DCs were generated by culturing $CD14^+$ monocytes in the presence of GM-CSF/IFN or GM-CSF/IL-4 for three days. At this time, DCs were pulsed with pooled HLA class I-restricted EBV peptides, and used to stimulate autologous PBLs. Cultures were restimulated with peptide-pulsed DCs at 7 and 14 days after the initial co-culture. ELISPOT assays were performed on unstimulated PBL, and after one or two round of stimulation, in order to evaluate the number of T cells producing IFN-γ after an overnight incubation with peptide-pulsed autologous monocytes (for donors FZ and FB) or T2 cells (TAP-/-, $HLA\text{-}A2^+$) (for donor LL). The results of the ELISPOT assays performed after two rounds of stimulation are shown in Figures 15A, 15B, 15C. In all the three donors, peptide-specific T cells were either undetectable or present at very low frequencies in unstimulated PBL cultures (day 0), depending on the peptide. As for donor LL, whereas a similar expansion of T cells specifically recognizing BMLF-1- and EBNA 3C-derived peptides was stimulated by IFN-DCs and IL-4-DCs, a significant increase in the frequencies of T cells specific for the CTL epitopes derived from LMP-2, EBNA 3A, and gp350 antigens was observed only in PBL cultures stimulated with IFN-DCs. As for donors FZ and FB, the frequency of IFN-γ-

producing T lymphocytes reactive against the selected EBNA 3A- or EBNA 3B-derived peptides was significantly higher in the cultures of PBLs stimulated with IFN-DCs as compared to IL-4-DC-stimulated PBLs.

The induction of cytotoxic T lymphocytes by IFN-DCs vs. IL-4-DCs was assessed for donor FB after three rounds of stimulation of purified autologous $CD8^+$ T cells with peptide pulsed DCs (Fig.16). Significantly higher levels of specific cytotoxic activity were exerted by cultures stimulated with IFN-DCs as compared to IL-4-DCs at both effector to target ratios tested (Fig.16). Moreover, lowering the effector to target ratio resulted in only a slight decrease of cytotoxicity in the cultures stimulated with IFN-DCs, whereas it caused a virtual abrogation of specific killing in the IL-4-DC-stimulated $CD8^+$ cultures (Fig. 16, right panel). The cultures of $CD8^+$ cells from donor FB were analyzed for the expression of CD27 and CD45RA markers, before and after stimulation with IL-4- or IFN-DCs (Fig. 17). A similar increase in the percentage of $CD8^+$ cells with a memory phenotype ($CD45RA^-$ $CD27^+$) was observed after two rounds of stimulation with peptide-pulsed IL-4-DCs (panel E) or IFN-DCs (panel F), with respect to unstimulated $CD8^+$ cells (panel B) and $CD8^+$ cells stimulated with unpulsed IL-4-DCs (panel C) or IFN-DCs (panel D). In the cultures stimulated with unpulsed DCs, the number of $CD8^+$ cells rapidly declined after the second stimulation, preventing their further stimulation and phenotypic analysis. Interestingly, increased levels of $CD45RA^-$ $CD27^+$ cells were maintained after the third stimulation of $CD8^+$ cultures with peptide-pulsed IFN-DCs (panel H), whereas a sharp decrease in the percentage of $CD8^+$ cells with memory phenotype was observed after three rounds of stimulation with peptide-pulsed IL-4-DCs (panel G). Collectively, these observations suggest that IFN-DCs

can efficiently promote *in vitro* the expansion and survival of EBV-specific memory $CD8^+$ cells acting as cytotoxic effectors.

We then evaluated whether EBV peptide-pulsed IFN-DCs could stimulate in vivo specific CD8+ T lymphocytes capable of inhibiting lymphomagenesis in SCID mice reconstituted with PBMCs from an EBV-positive donor (29, 30). SCID mice were reconstituted with 4×10^7 PBMCs from donor FB. Three hours after reconstitution, the mice were divided into three groups. The first group of mice received no further treatment, whereas mice in the second and third group were injected i.p. with, respectively, 2 x 106 unpulsed IFN-DCs or IFN-DCs pulsed with a pool of EBNA 3A- and EBNA 3B-derived peptides. These peptides were the same used for in vitro stimulations of donor FB PBL. Seven days later, a boost dose (2×10^6 cells) of unpulsed or peptide-pulsed IFN-DCs was injected i.p. in the second and third group of mice, respectively. The DCs utilized in this experiment were derived from CD14+ monocytes obtained from donor AB, the identical twin of donor FB. There were five mice per group.

AS shown in table 2 below, vaccination of the reconstituted animals with autologous peptide-pulsed IFN-DCs caused a highly significant prolongation of survival time as compared to what observed for unvaccinated SCID mice and for mice vaccinated with unpulsed IFN-DCs (Table 2). Overall, these results indicate that IFN-DCs are efficient in stimulating in vivo the expansion of effector CD8+ T lymphocytes.

Table 2

Vaccine	Mean time of death (± SD)		
None	58.2 (±9.7)	NS	$p < 0.001$
Unpulsed IFN-DCs	65.0 (±16.9)	$p < 0.03$	
Peptide-pulsed IFN-DCs	90.6 (±2.3)		

Example 11: Generation of dendritic cells from total PBMC.

The ability of IFN-α to differentiate dendritic cells (DC) from total peripheral blood mononuclear cells (PBMC) was evaluated using the VacCell® technology (IDM, Paris, France). The cells differentiated in presence of IFN-α are referred as IFN-DC. Two sources of IFN-α were tested: natural IFN-α (Interferon Sciences) and recombinant IFN-α (Pepro Tech).

1) Material and methods

Leucocytes from healthy donors were isolated from peripheral blood by cytapheresis. Cells were washed three times by centrifugation at 500 rpm using the washing buffer included in the VacCell® cell processor (PBS glucose without magnesium nor calcium).

PBMCs were seeded in EVA (Ethylene Vinyl Acetate) bags at 5.106 cells/ml (STEDIM, 60 ml) in AIMV modified medium (Invitrogen) in presence of GM-CSF (Leucomax, Novartis, 500 IU/ml) and IFN-α. For 3 healthy donors, two concentrations of IFN-α were tested (5.000 and 1.000 IU/ml), and for 2 healthy donors only 5000 IU/ml of IFN-α were used. After 3 days of culture at 37°C, cells were washed as previously described.

Cell morphology in culture was examined and compared with morphology at day 0. Cell recovery was determined by Malassez counting, and cell viability by Trypan Blue or TO-PRO3 staining.

Cells were phenotyped with monoclonal antibodies coupled to fluorochromes (Beckmann Coulter Immunotech, Marseille) for CD14 (clone RM052), CD1a, CD80 (clone MAB104), CD83 (clone HB15a), CD86 (HA5.2B7), HLA-ABC (B9.12.1), HLA-DR (B8.12.2) CD40 (clone MAB89) and CD25 (B1.19.9), surface expression was measured using dead cells exclusion by TO-PRO3 staining.

Part of the of the IFN-DCs obtained were maturated using the following conditions: 1.106 cells/well (0.5 ml), containing 100µg/ml poly I:C and 3µg/ml anti-CD40 in 24 wells plates and cultured for 40 hours. Maturation phenotype was determined.

IL-12 and IL-10 secretions were evaluated in supernatants by ELISA using reagents from R & D.

Cells were tested for their capacity to present allo-antigens in MLR assays performed according to the following:

Viable IFN-DCs were re-suspended in complete medium (AIMV, 1% antibiotic P.S, 5% AB human serum) at a density of 1.10^6 cells/ml (falcon conical 15 ml tube) containing mitomycin C to a final concentration of 50µg/ml, for 30 minutes at 37°C. IFN-DCs were washed three times with AIMV and re-suspended in complete medium at the appropriate density: ratio of 1/3 (0.33 10^6 cells/ml), 1/10, 1/30, 1/100 and 1/300. Allogenic T cells were adjusted to a concentration of $1x10^6$ cells/ml in complete medium. 1x105 T cells (100µl) were seeded in 96 conical bottom plates then 100µl of the different dilution of IFN-DCs were added.The control conditions were IFN-DCs alone, T cells alone (negative controls) and T cells + 1µg/ml of PHA (completed

with 100µl complete medium). At day 4, 3H-thymidine was added at a concentration of 100µCi/mL in AIMV + 5% AB human serum (10µl/well = 1µCi/well) and left at 37°C for 18 hours. At day 5, the radioactivity was counted.

2) Results:

The following parameters were evaluated after 3 days of differentiation:

a) Morphology:

After 3 days of differentiation, IFN-DCs morphology was characteristic of that of dendritic cells.

b) Differentiation yield of monocytes into IFN-DCs:

They were evaluated by comparing, for each culture condition, the percentage of DCs after differentiation to the percentage of monocytes in the blood sample. The results obtained are shown in table 3 below.

Table 3

	Culture yield (%, at day 3)
GMCSF + recombinant IFNα 1000U/ml	*95.4*
GMCSF + recombinant IFNα 5000U/ml	*88.7*
GMCSF + natural IFNα 1000U/ml	*95.4*
GMCSF + natural IFNα 5000U/ml	*78.7*

The percentage of cells recovery ranges from very high (95 %) to high (close to 80%) according the dose of IFN used. These results are somewhat better than those obtained with purified cells, suggesting a possible preserving effect exerted by contaminating cells.

c) Cell viability:

After maturation, DCs viability has been evaluated by TO-PRO3 staining and ranged from about 78 to about 92 %. As shown in table 4 below, the viability of DCs was superior to 90 %. Cell viabilities were equivalent whatever the source of IFNα.

Table 4

	Viability (%, trypan blue)	Viability (% of negative topro-3)
	Day 0	Day 3
GMCSF + recombinant IFNα 1000U/ml	95	Lymphocytes: 94% DCs: 97%
GMCSF + recombinant IFNα 5000U/ml	95	Lymphocytes: 96% DCs: 94%
GMCSF + Natural IFNα 1000U/ml	95	Lymphocytes: 97% DCs: 94%
GMCSF + Natural IFNα 5000U/ml	95	Lymphocytes: 98% DCs: 94%

d) Surface marker expression

Expression of CD14, CD83 and HLA-DR are shown on Figure 18. The general phenotypic characterization of the cells is summarized in the following table 5:

Table 5

Surface marker	CD14	CD1a	CD25	CD80	CD83	CD86	HLA-ABC	HLA-DR
IFN-DC	low	++	-	++	-/low	++	++	++
Maturated IFN-DC	-	++	+	ND	low/+	+++	++	++

e) IL-12 and IL-10 secretion (results are expressed for one donor in pg/ml)

After 3 days of culture, cells were washed and contacted, at a concentration of 2.10^6 cells / ml for 24 hours with a culture medium containing or not maturation agents.IL-10 and IL-12 secretion was assessed by classical ELISA.

	Immature DCs		Mature DCs	
	Native IFN	Recombinant IFN	Native IFN	Recombinant IFN
IL-12 secretion	30	25	15	10
IL-10 secretion			240	190

Immature IFN-DCs secreted only IL-12 and not IL-10. Maturation with polyIC and anti-CD40 induced an IL-10 secretion by IFN-DCs in all donors. In these experiments, IL-12 secretion appears to be relatively weak for IFN-DCs obtained in three days. The results obtained represent a specific time point observation of the secretion of non-purified cells.

f)Ability of immature or mature IFN-DCs to present allo-antigens evaluated in mixed lymphocyte reactions (MLR)

Immature IFN-DCs are able of efficient allo-antigen presentation to T cells. This capacity augments when IFN-DCs are further matured (Figure 19).

These experiments demonstrate that DCs defined by morphology, surface marker expression and ability for

efficient allo-antigen presentation could be differentiated from PBMC in presence of GM-CSF and IFN-α in 3 days of culture. IFN-DCs obtained had the ability to further mature phenotypically under treatment with polyIC and anti-CD40. No systematic differences were observed between the two concentrations of IFN-α tested.

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CLAIMS:

1. A process for the preparation of dendritic cells comprising the step of culturing mononuclear cells in a culture medium containing type I interferon, wherein said mononuclear cells are chosen from the group consisting of total peripheral blood mononuclear cells, adherent peripheral blood mononuclear cells and highly purified CD^{14+} monocytes isolated from peripheral blood mononuclear cells.
2. A process according to claim 1, wherein said dendritic cells are obtained in no more than three days.
3. A process according to claim 1 or 2, wherein said type I interferon is chosen from the group consisting of natural or recombinant IFNα, natural or recombinant INFβ consensus interferon and any synthetic type I interferon.
4. A process according to claim 1 to 3, wherein the concentration of said type I interferon in the culture medium is greater than 100 IU/ml.
5. A process according to claim 4 wherein said concentration is between 100 and 10.000 IU/ml.
6. A process according to claim 5 wherein said concentration is between 400 and 10.000 IU/ml.
7. A process according to claim 6 wherein said concentration is between 500 and 2.000 IU/ml.
8. A process according to claim 7 wherein said concentration is about 1000 IU/ml.
9. A process according to claims 1 to 8 wherein said culture medium also contains a cell growth factor.
10. A process according to claim 9 wherein said cell growth factor is GM-CSF.

11. A process according to claim 10 wherein said the concentration of said GM-CSF in the medium is between 250 and 1000 IU/ml.

12. A process according to claims 1 to 11 wherein said process further comprises the step of contacting the dendritic cells obtained with a maturation agent.

13. Dendritic cells obtainable with a process according to claims 1 to12.

14. Dendritic cells according to claim 13 said cells having been loaded with antigenic peptides or proteins, with a cellular extract containing at least one antigen or with nucleic acid molecules encoding for antigens to which an immune response is of interest

15. Dendritic cells according to claim 13 or 14 wherein said cells are in a dehydrated or frozen form in an appropriate cryo-preservative medium..

16. A kit for preparing dendritic cells according to claim 13 comprising:

a) single use elements necessary for the culture and the washing of the cells;

b) a composition comprising type I IFN and compatible additives;

c) optionally a composition comprising a cell growth factor and compatible additives; and

d) optionally a composition comprising antigens or nucleic acids encoding for antigens to which an immune response is of interest.

17. A pharmaceutical composition or a vaccine comprising, as an adjuvant, the dendritic cells according to any one of claims 13 to 15 together with at least one immunogen and a pharmaceutically acceptable vehicle or an auxiliary agent.

18. A pharmaceutical composition or a vaccine comprising, as an active principle, the dendritic cells according to any one of claims 13 to 15 together with a pharmaceutically acceptable vehicle or auxiliary agent.

19. A pharmaceutical composition or a vaccine according to claim 18 wherein said dendritic cells are dendritic cells according to claim 15.

20. Use of dendritic cells according to any one of claims 13 to 15 for the preparation of a vaccine or a pharmaceutical composition for the prevention or the treatment of a pathology associated with the presence of an antigen in the human body.

21. Use according to claim 20 wherein said pathology is an infectious or neoplastic disease.

22. Use according to claim 21 wherein said infectious disease is a viral infection.

23. Use according to claim 22 wherein said viral infection is a HIV, a HBV or a HCV infection.

24. Use according to claim 20 wherein said neoplastic disease is a lymphoma.

25. Use according to claim 21 or 24 wherein said neoplastic disease is virally induced.

26. Use according to claim 25 wherein said neoplastic disease is induced by Epstein-Barr virus.

27. Use according to claims 20 to 26 wherein said pharmaceutical composition is suitable for administration at the site of infection or within the tumour.

28. 28. A method for the ex-vivo expansion of T cells, comprising the step of putting in contact said T cells with the dendritic cells according to any one of claims

13 to 15.A pharmaceutical composition containing, as active principle, the T cells according to claim 28.

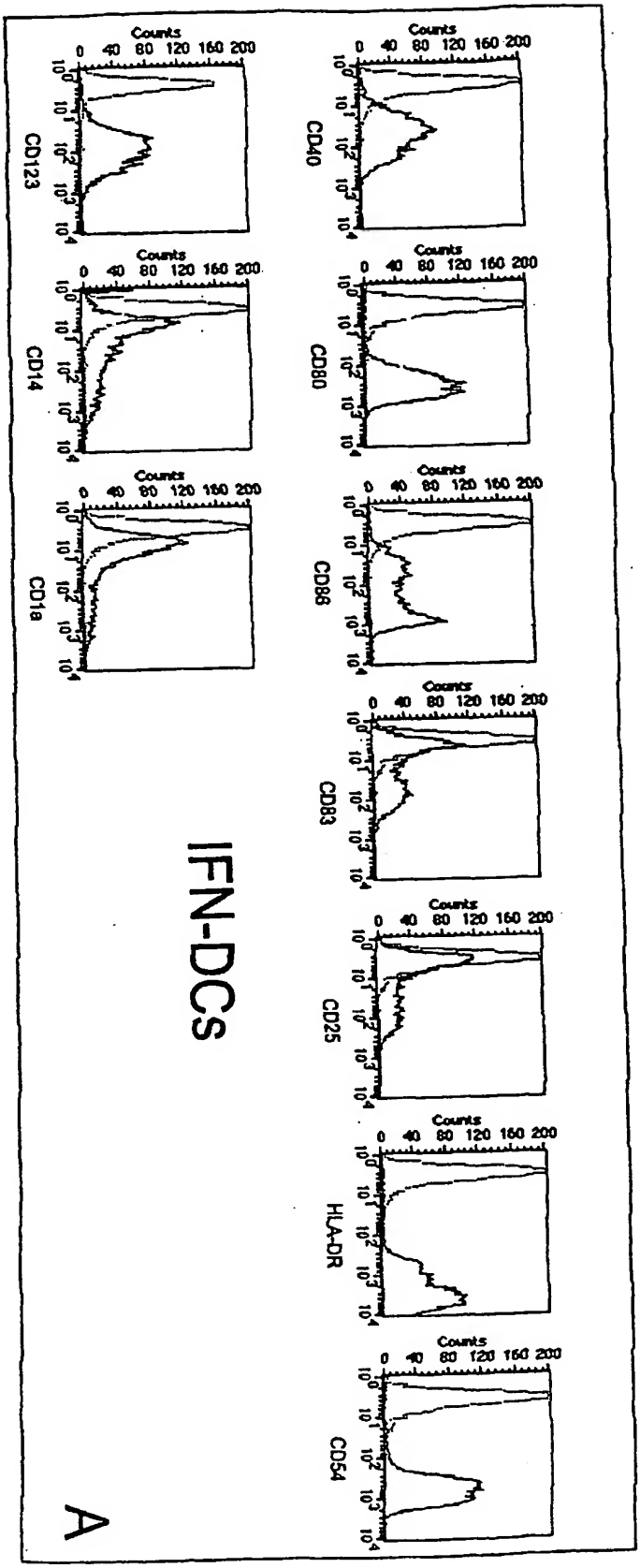

IFN-DCS
CD123
CD14
CD1a
CD40
CD80
CD86
CD83
CD25
HLA-DR
CD54
Counts
0 40 80 120 160 200
A

Fig. 1A

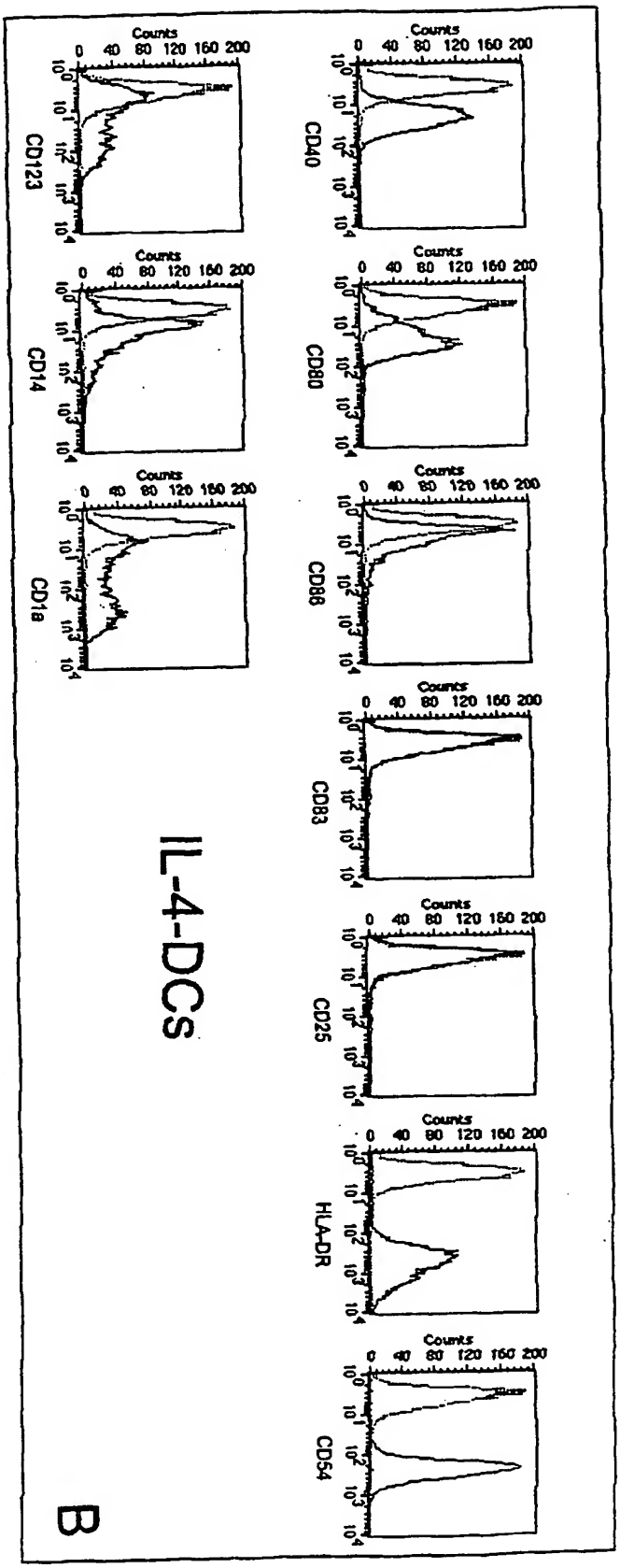
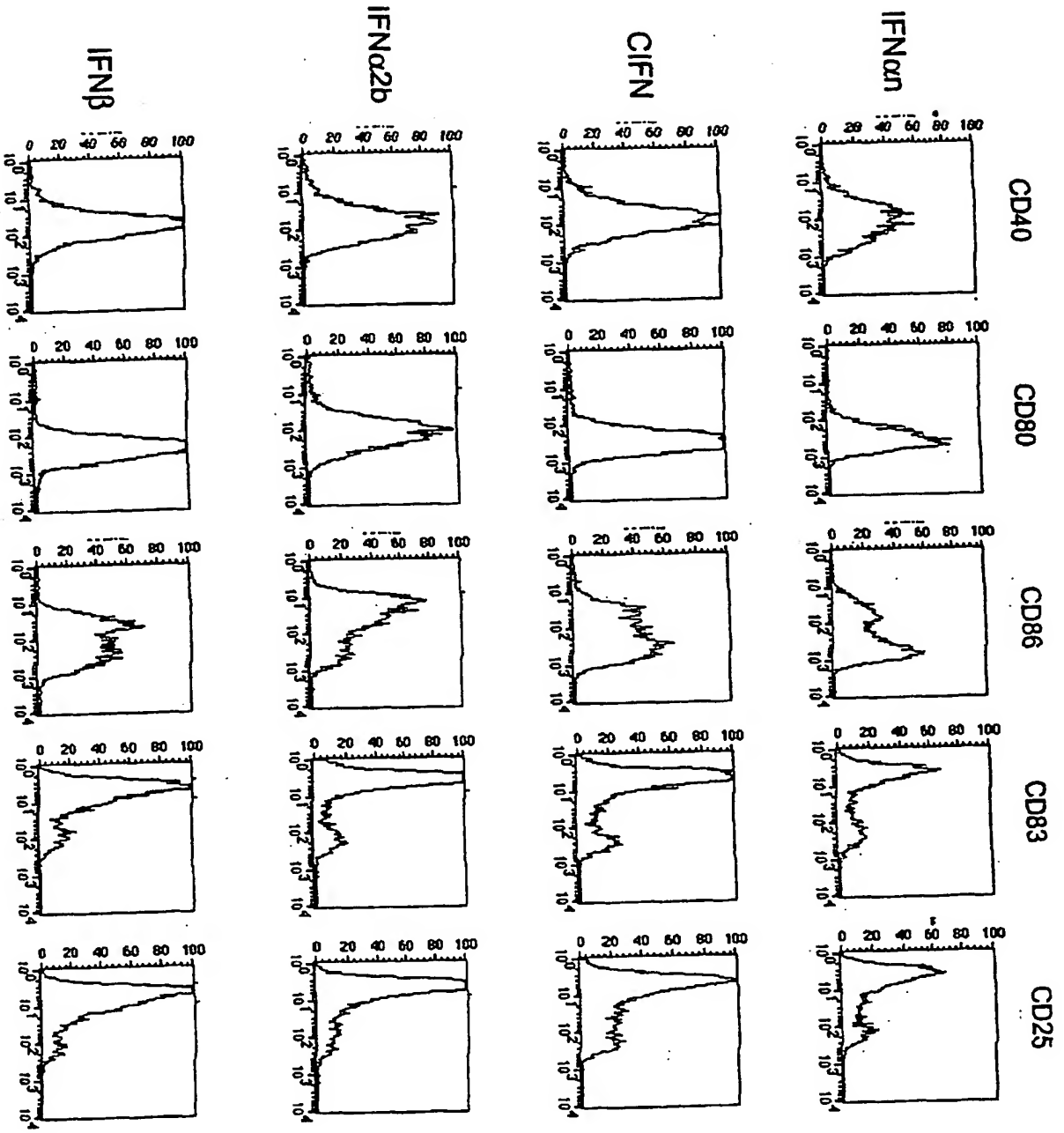

B
IL-4-DCs
CD123
CD14
CD1a
CD40
CD80
CD86
CD83
CD25
HLA-DR
CD54
Counts
0 40 80 120 160 200

Fig. 1B

Fig.2



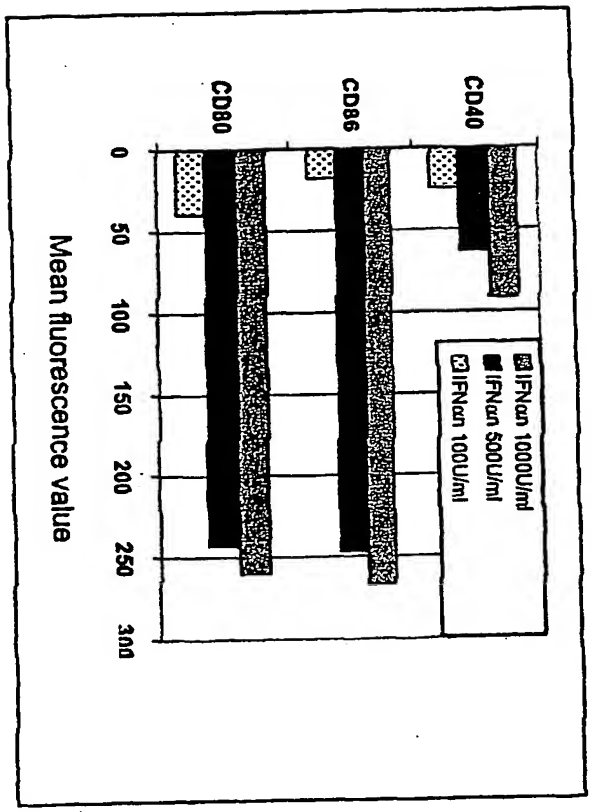

CD80
CD86
CD40
0
50
100
150
200
250
300
Mean fluorescence value
IFNαn 1000U/ml
IFNαn 500U/ml
IFNαn 100U/ml

Fig. 3

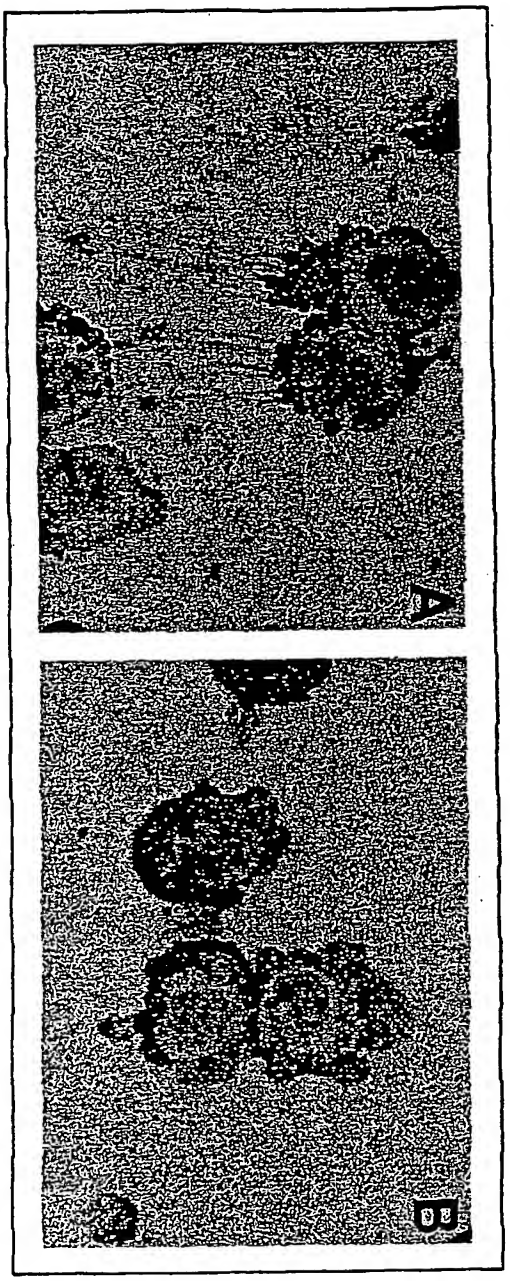


Fig. 4

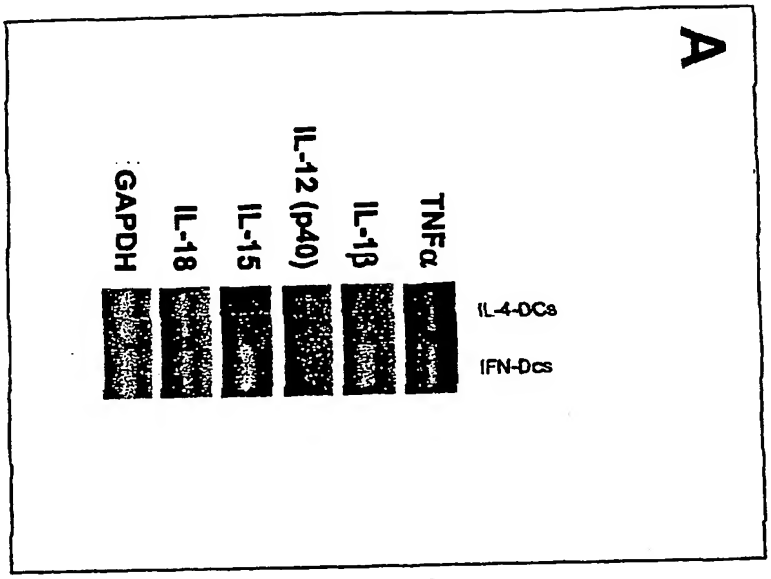

A
TNFα
IL-1β
IL-12 (p40)
IL-15
IL-18
GAPDH
IL-4-DCs
IFN-Dcs

Fig. 5A

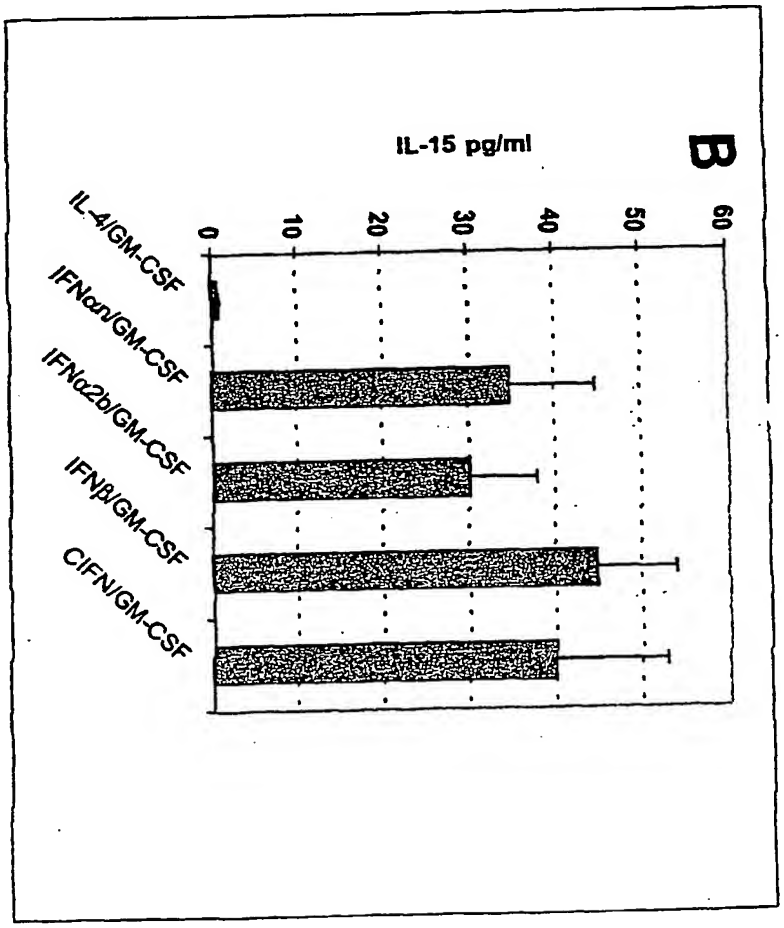

B
IL-15 pg/ml
0
10
20
30
40
50
60
IL-4/GM-CSF
IFNαn/GM-CSF
IFNα2b/GM-CSF
IFNβ/GM-CSF
CIFN/GM-CSF

Fig. 5B

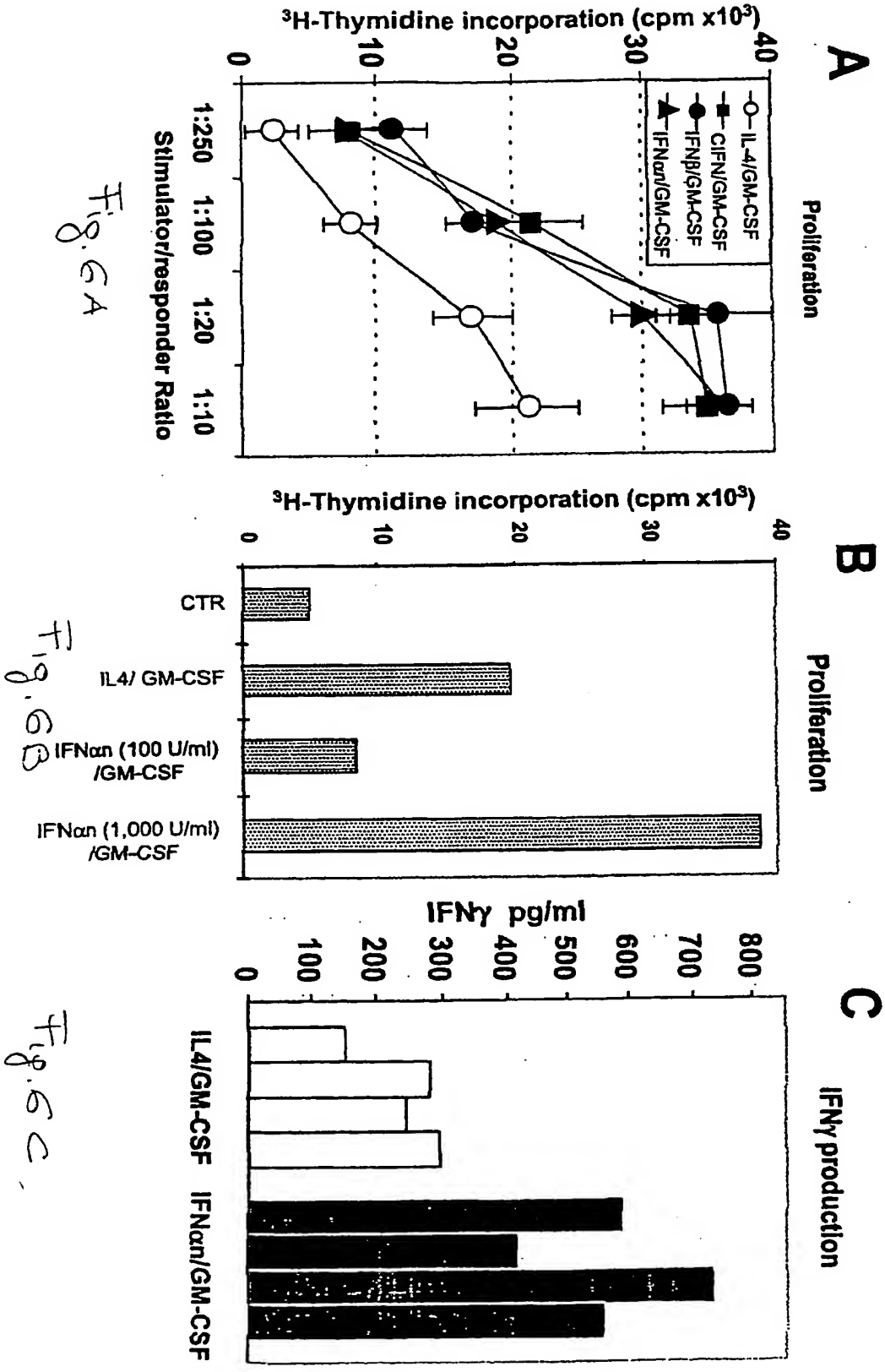


Fig. 6A

Fig. 6B

Fig. 6C

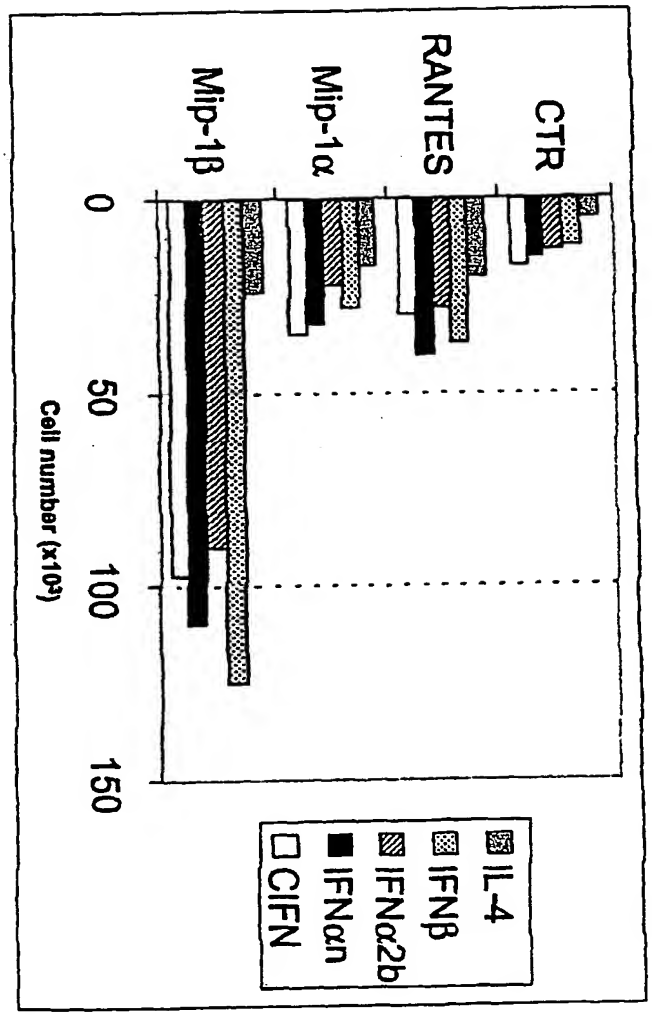

CTR
RANTES
Mip-1α
Mip-1β
0
50
100
150
Cell number (x10³)
IL-4
IFNβ
IFNα2b
IFNαn
CIFN

Fig. 4

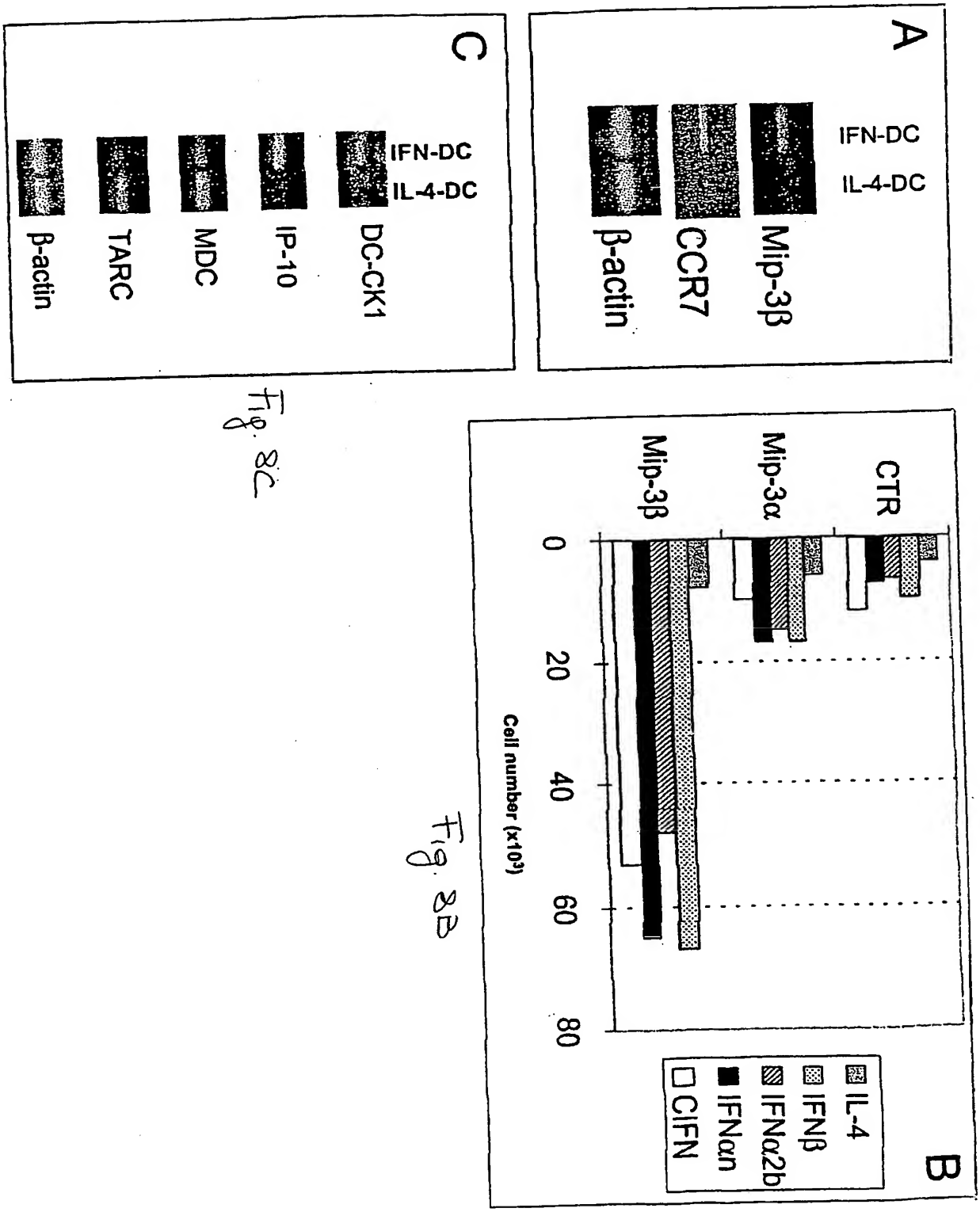
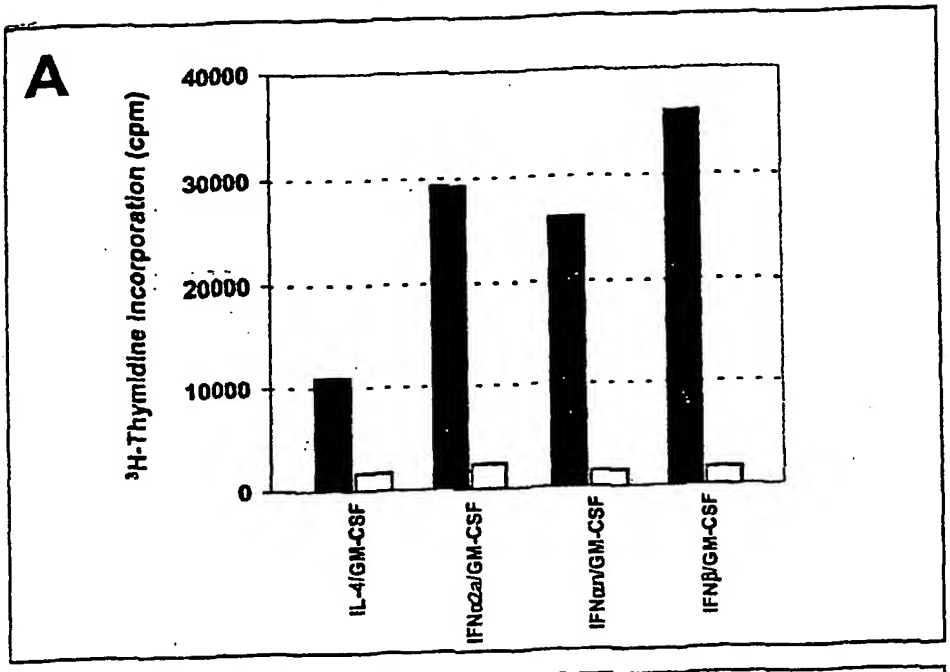


Fig. 8A

Fig. 8C

Fig. 8B

Fig.9A


A
^{3}H-Thymidine incorporation (cpm)
40000
30000
20000
10000
0
IL-4/GM-CSF
IFNα2a/GM-CSF
IFNαn/GM-CSF
IFNβ/GM-CSF

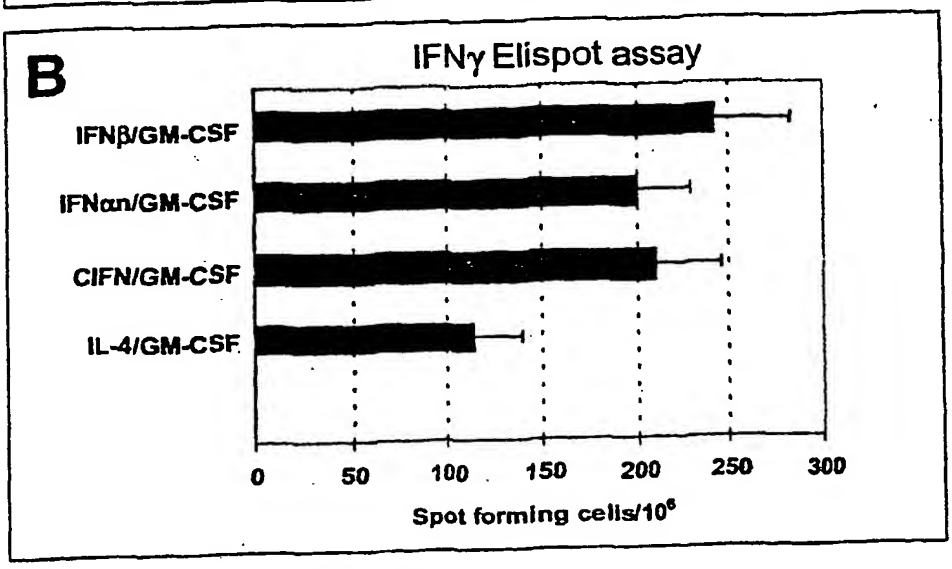
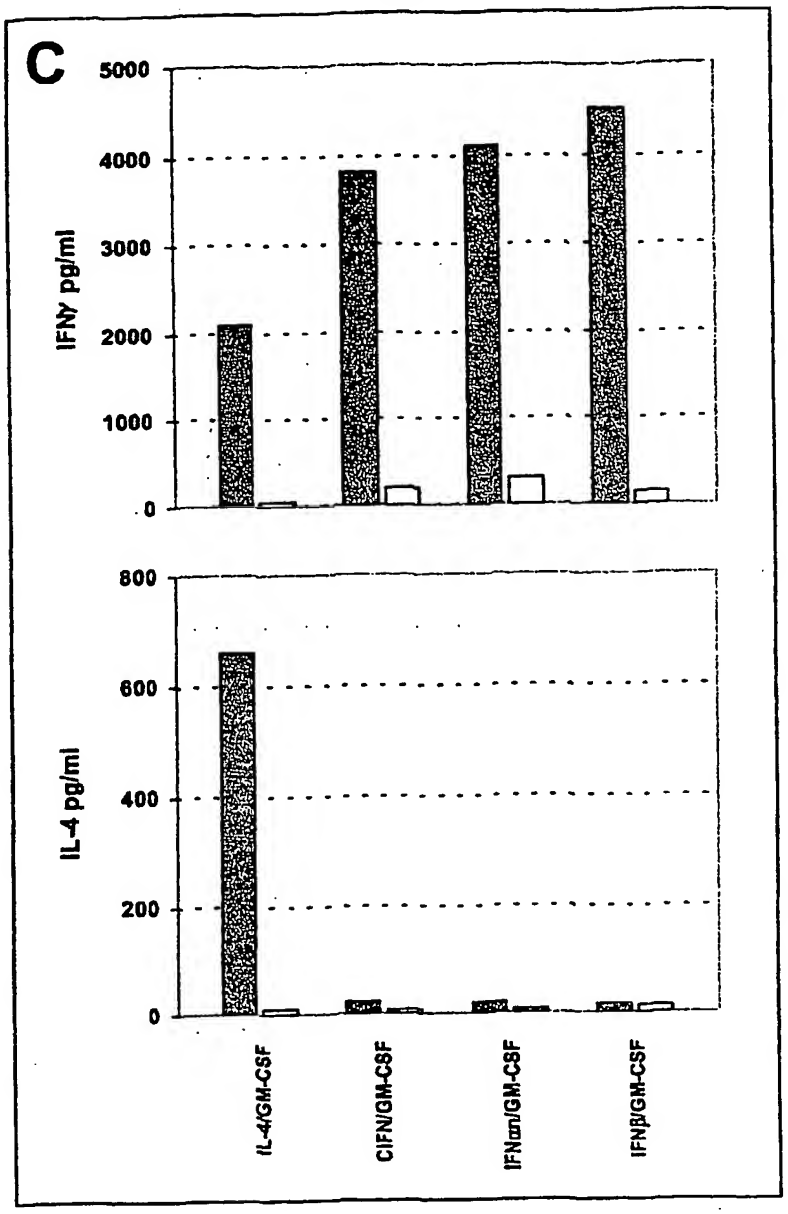

B
IFNγ Elispot assay
IFNβ/GM-CSF
IFNαn/GM-CSF
CIFN/GM-CSF
IL-4/GM-CSF
0
50
100
150
200
250
300
Spot forming cells/10^6

Fig.9B

Fig. 9c


C
IFNγ pg/ml
5000
4000
3000
2000
1000
0
IL-4 pg/ml
800
600
400
200
0
IL-4/GM-CSF
CIFN/GM-CSF
IFNαn/GM-CSF
IFNβ/GM-CSF

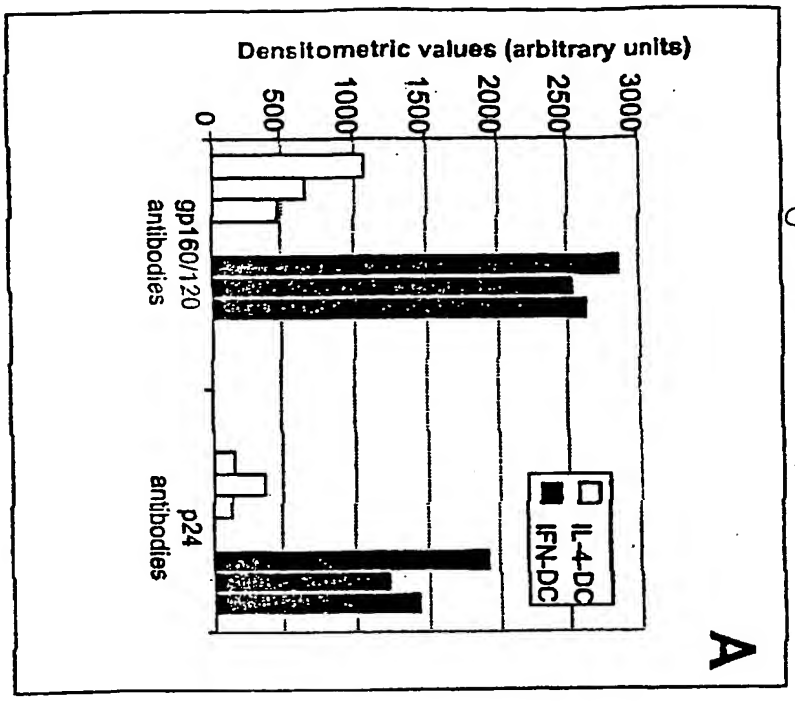


Fig. 10 A

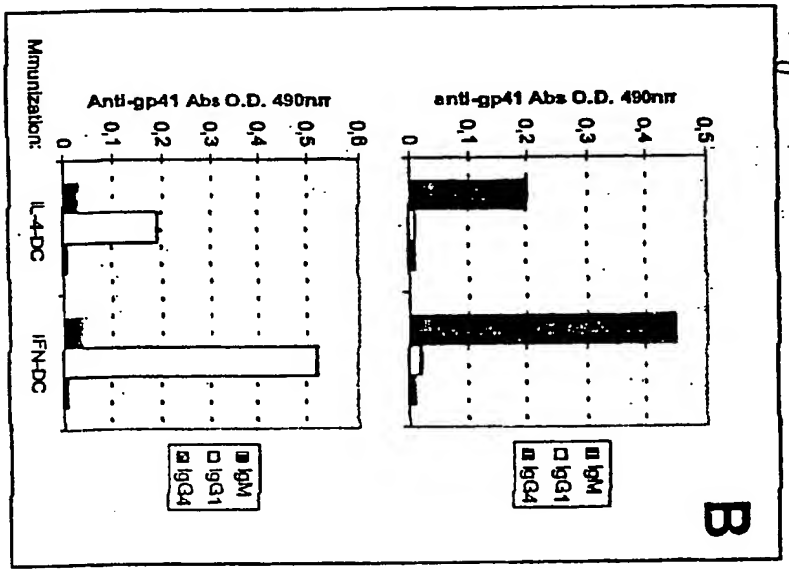


Fig. 10 B

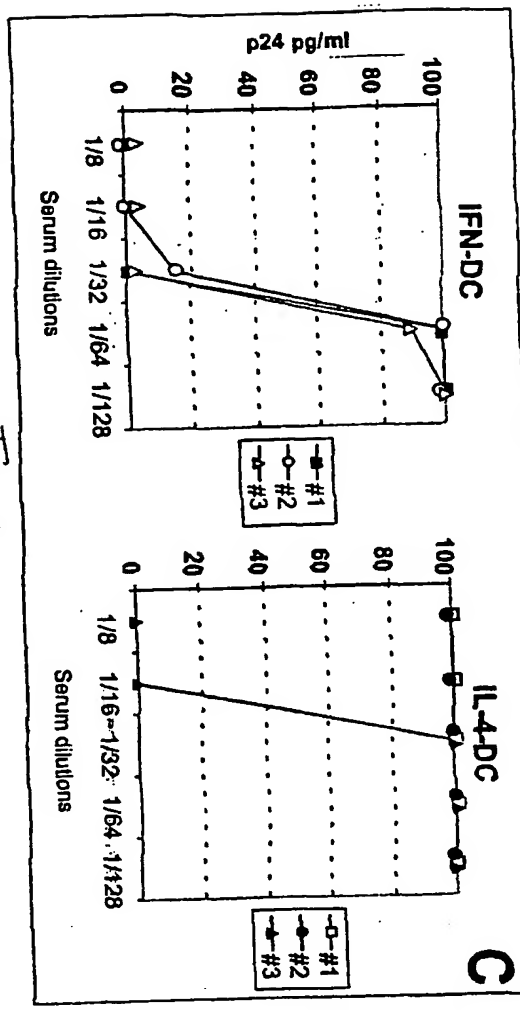


Fig. 10C

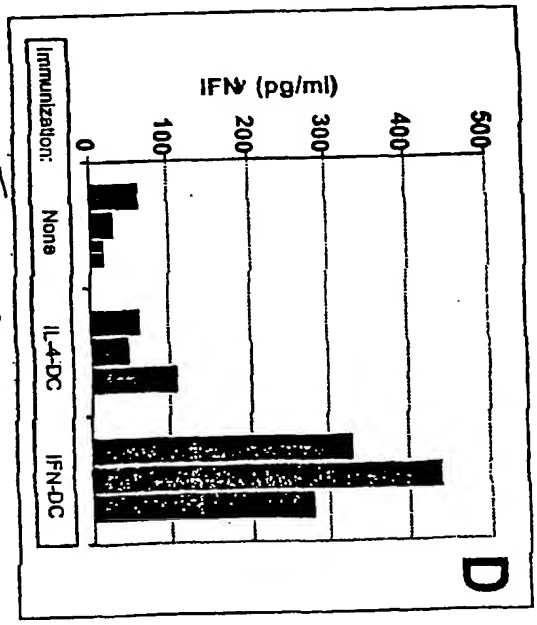


Fig 10D

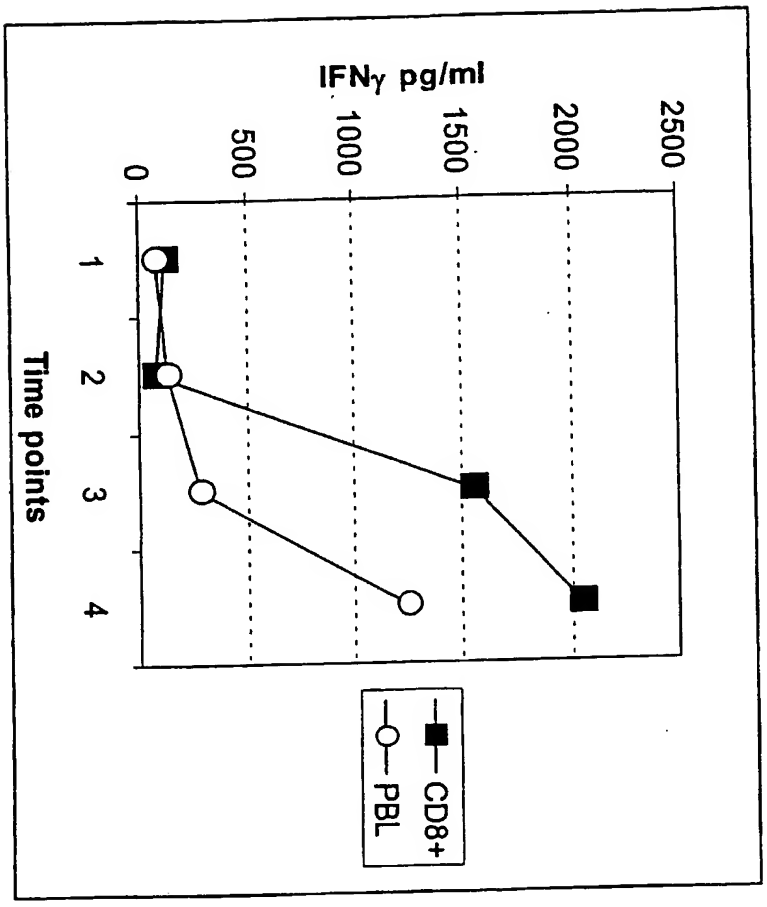


Fig. 11

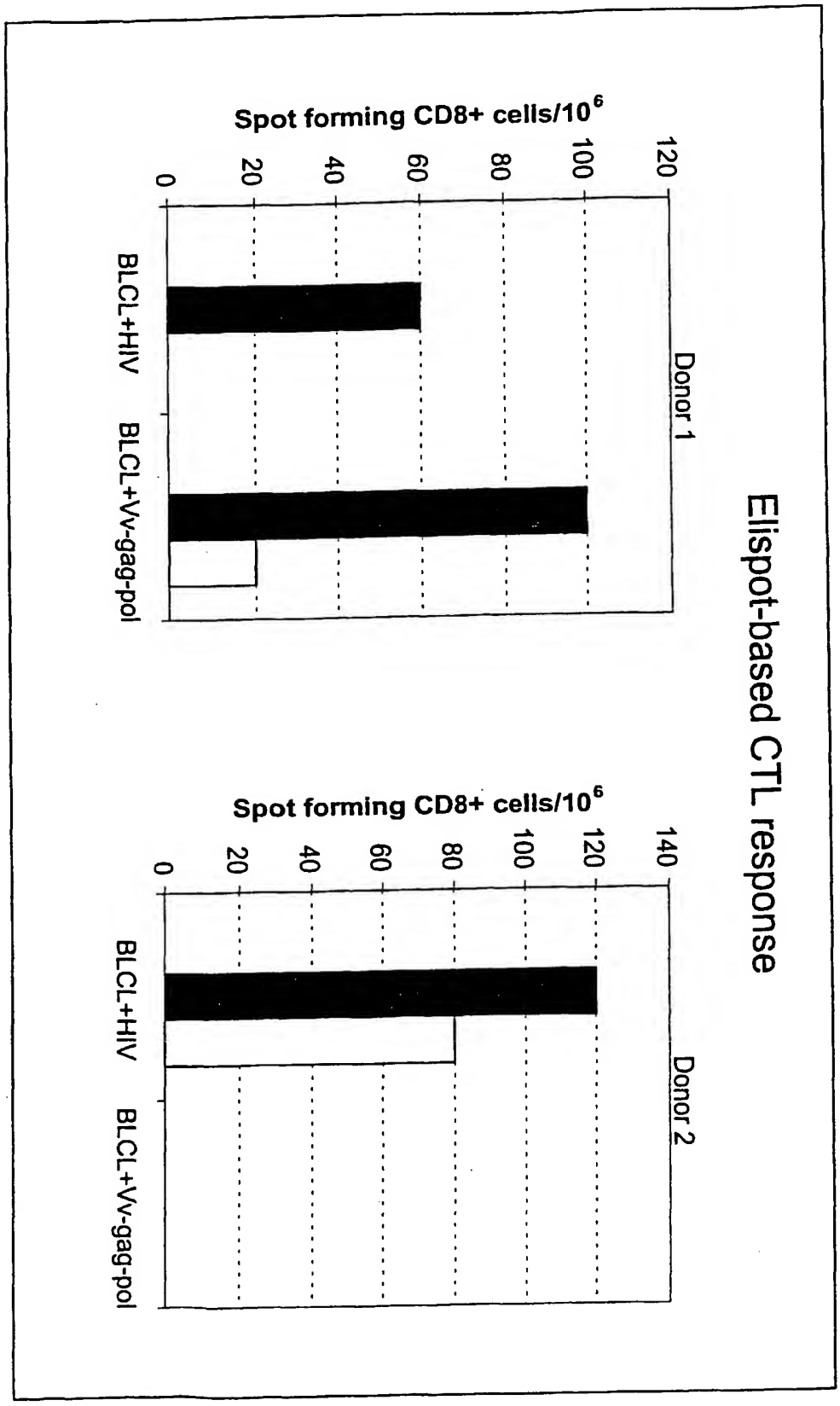

Elispot-based CTL response
Spot forming CD8+ cells/10^6
0
20
40
60
80
100
120
BLCL+HIV
BLCL+Vv-gag-pol
Donor 1
Spot forming CD8+ cells/10^6
0
20
40
60
80
100
120
140
BLCL+HIV
BLCL+Vv-gag-pol
Donor 2

Fig. 12

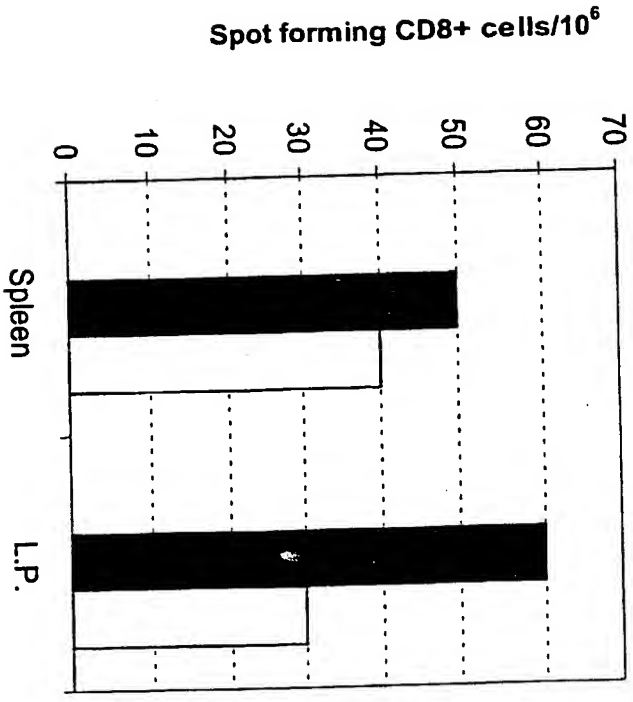

Elispot-based response to the HLA-A*0201-restricted CTL epitope SL9
Spot forming CD8+ cells/10^6
0
10
20
30
40
50
60
70
Spleen
L.P.

Fig. 13

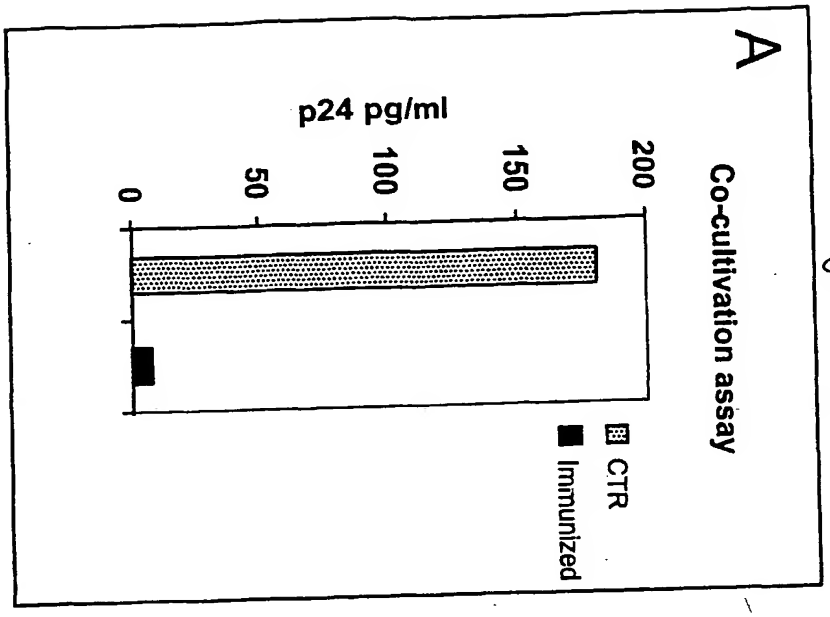


Fig. 14A

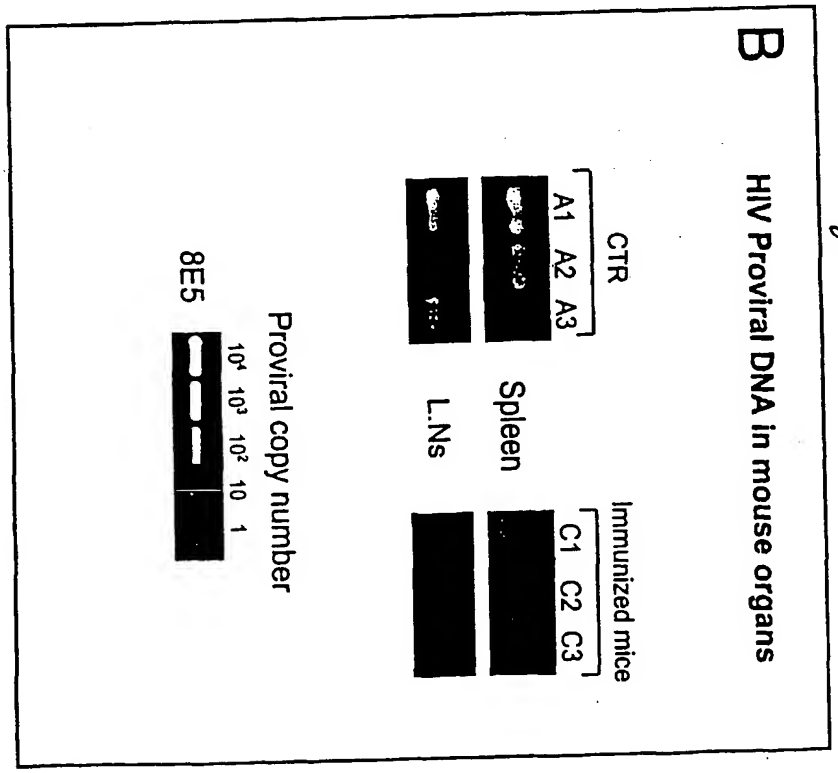


Fig. 14B

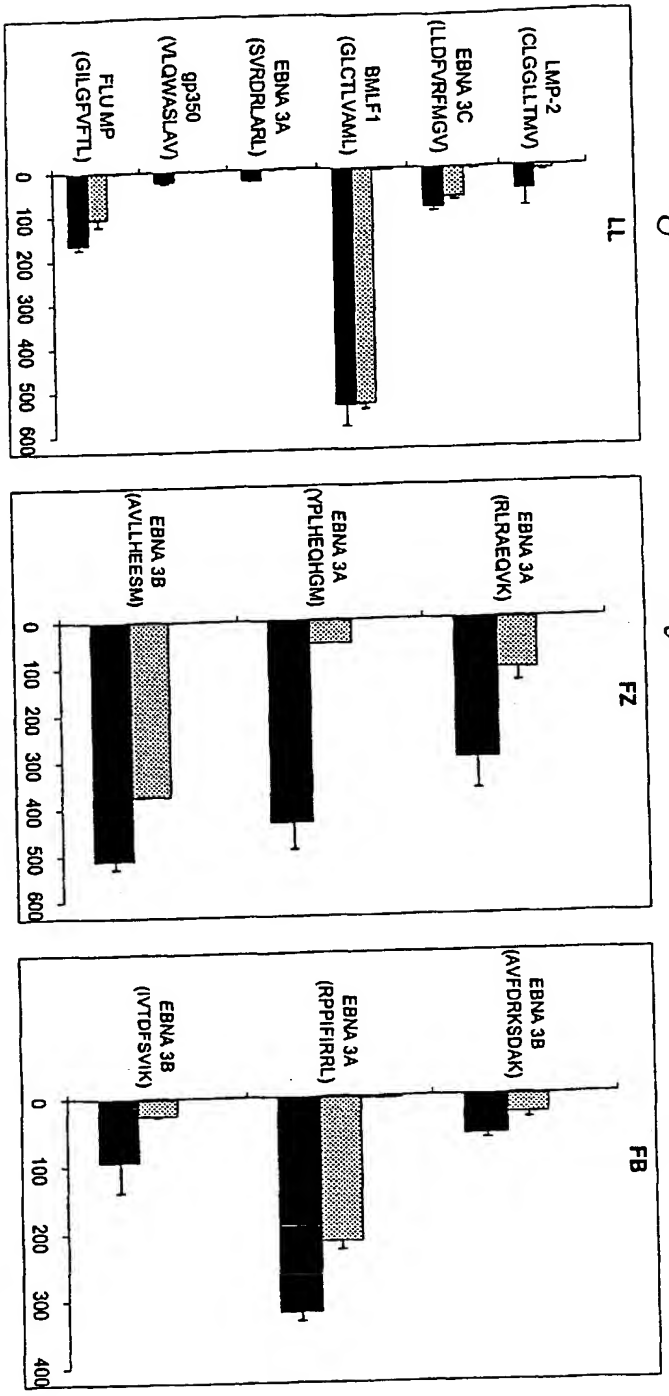


Fig. 15A

Fig. 15B

Fig. 15C

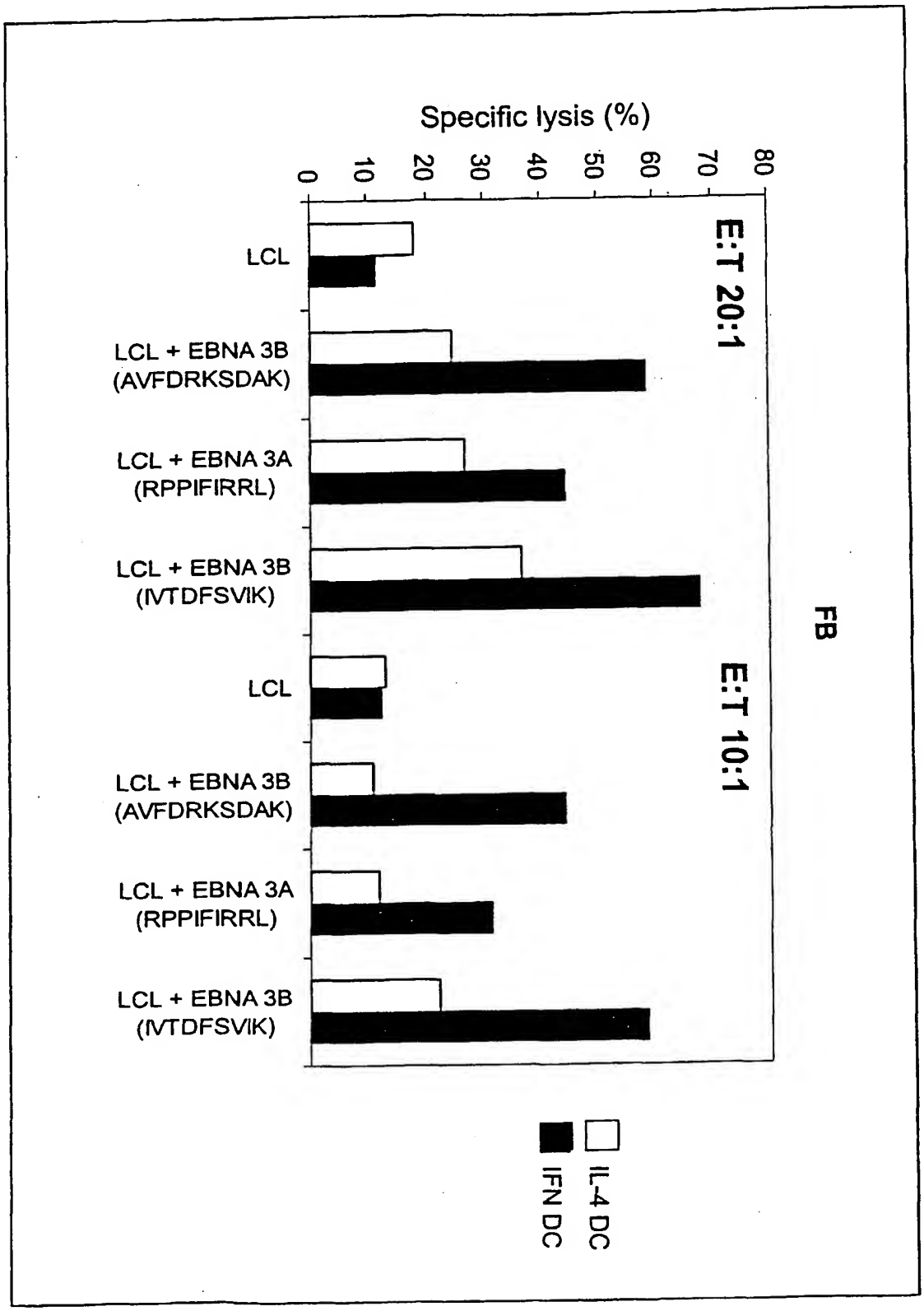

Specific lysis (%)
0
10
20
30
40
50
60
70
80
E:T 20:1
E:T 10:1
LCL
LCL + EBNA 3B (AVFDRKSDAK)
LCL + EBNA 3A (RPPIFIRRL)
LCL + EBNA 3B (IVTDFSVIK)
LCL
LCL + EBNA 3B (AVFDRKSDAK)
LCL + EBNA 3A (RPPIFIRRL)
LCL + EBNA 3B (IVTDFSVIK)
IFN DC
IL-4 DC
FB

Fig. 16

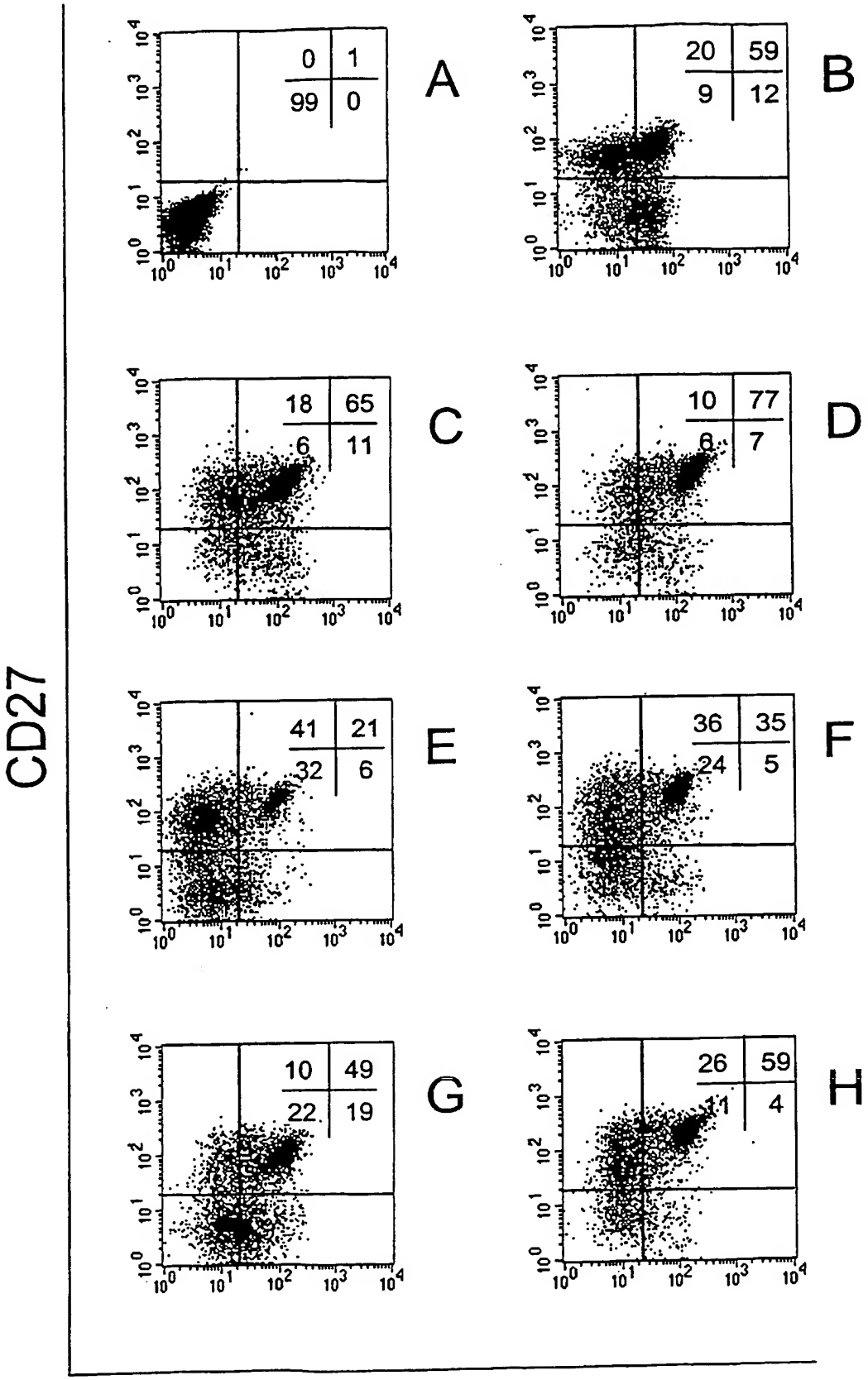

CD27
CD45RA
A
0
1
99
0
B
20
59
9
12
C
18
65
6
11
D
10
77
6
7
E
41
21
32
6
F
36
35
24
5
G
10
49
22
19
H
26
59
11
4

Fig. 17

Fig. 18.A

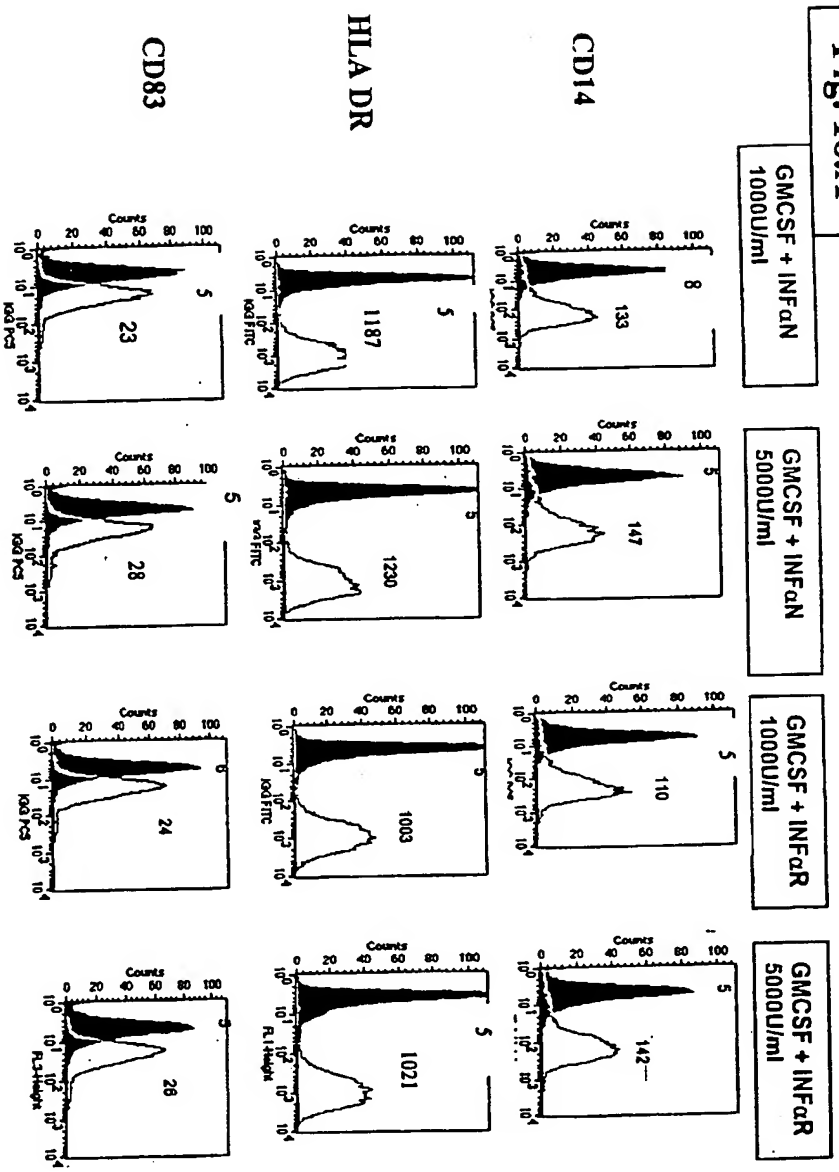
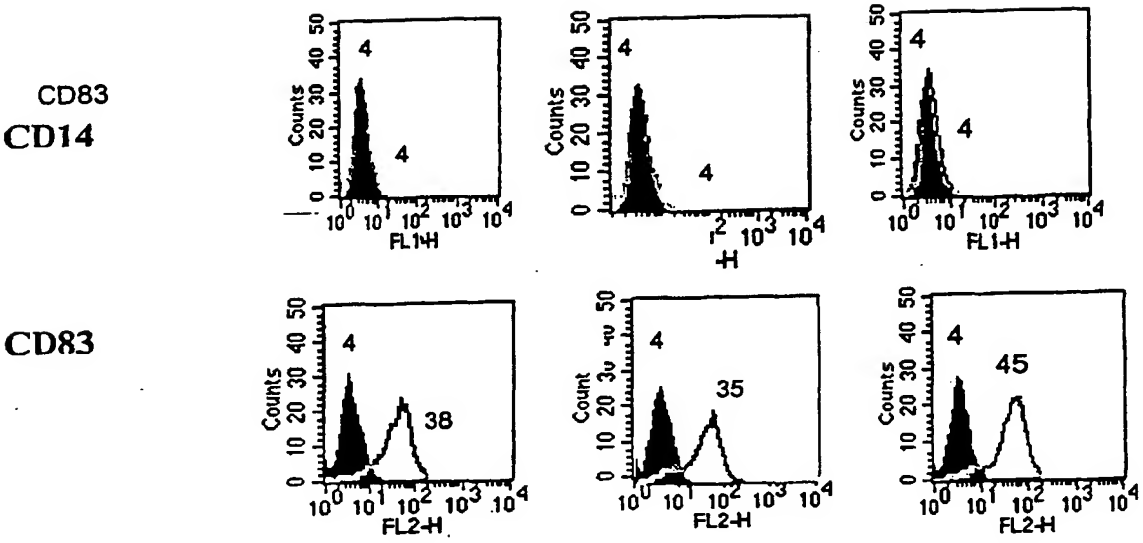

GMCSF + INFαN 1000U/ml
GMCSF + INFαN 5000U/ml
GMCSF + INFαR 1000U/ml
GMCSF + INFαR 5000U/ml
CD14
HLA DR
CD83
Counts
133
147
110
142
1187
1230
1003
1021
23
28
24
28

Fig. 18.B



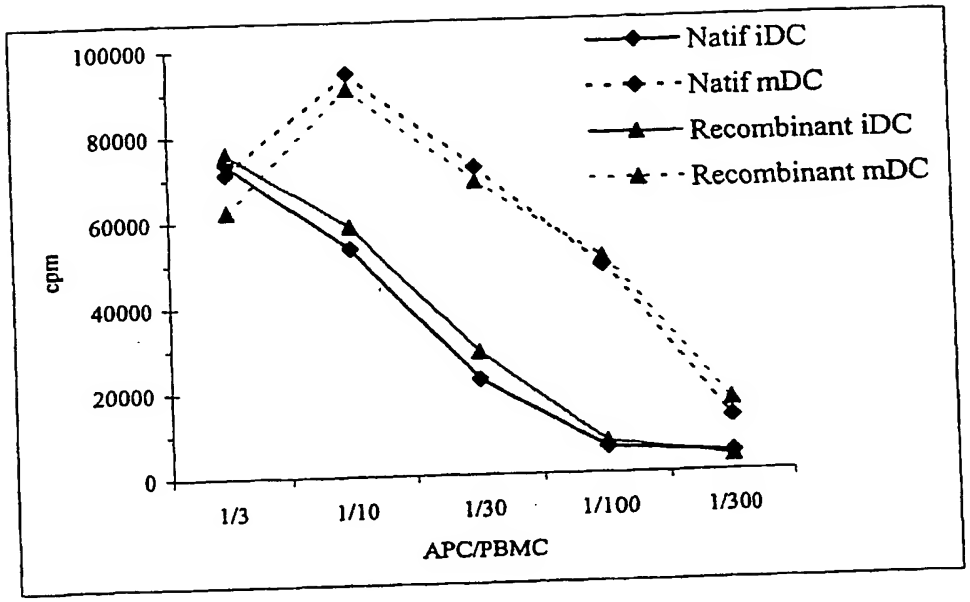

Natif iDC
Natif mDC
Recombinant iDC
Recombinant mDC
100000
80000
60000
40000
20000
0
cpm
1/3
1/10
1/30
1/100
1/300
APC/PBMC

Fig. 19

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(71) Applicant *(for all designated States except US)*: **ISTITUTO SUPERIORE DI SANITÀ** [IT/IT]; Viale Regina Elena, 299, I-00161 Roma (IT).

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(74) Agent: **GERLI, Paolo**; Notarbartolo & Gervasi, Corso di Porta Vittoria, 9, I-20122 Milan (IT).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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(54) Title: METHOD FOR GENERATING HIGHLY ACTIVE HUMAN DENDRITIC CELLS FROM MONOCYTES

(57) Abstract: The present invention relates to a process for deriving dendritic cells from mononuclear cells in culture comprising the step of putting in contact type I IFN with said mononuclear cells. Dendritic cells suitable as cellular adjuvants in prophylactic as well as therapeutic vaccination of animal and human beings, are obtainable thereby, after a single step treatment in a brief period of time. Dendritic cells obtainable thereby, pharmaceutical compositions including them, in particular a vaccine comprising said cells as active principle, and a method of treatment of a pathology associated with the presence of an antigen in human beings, are further objects of the invention, as well as a kit for deriving said dendritic cells and a method for the ex vivo expansion of T cells using them.

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A61P31/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SANTINI M ET AL: "Type I Interferon as a Powerful Adjuvant for Monocyte-Derived Dendritic Cell Development and Activity In Vitro and in Hu-PBL-SCID Mice" JOURNAL OF EXPERIMENTAL MEDICINE, TOKYO, JP, vol. 191, no. 10, 15 May 2000 (2000-05-15), pages 1777-1788, XP002182907 ISSN: 0022-1007	1-14, 16-18, 20-23,28
Y	abstract page 1778, column 1, paragraph 3 -column 2, paragraph 1 page 1779, column 1, paragraph 4 page 1779, column 2, last paragraph -page 1181, column 2, paragraph 1 page 1783, column 1, last paragraph -page 1785, column 1, line 6 -/--	15,19, 21,24-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2003	06/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, V

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	page 1785, column 2, paragraph 2 page 1786, column 1, line 29 - line 34	
X	PAQUETTE RONALD L ET AL: "Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells." JOURNAL OF LEUKOCYTE BIOLOGY, vol. 64, no. 3, 1998, pages 358-367, XP008014325 ISSN: 0741-5400 the whole document	1,3-6, 9-11,14, 16,28
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat[redacted]pplication No
PCT/EP 02/04709

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